BIOCHEMICAL STORAGE PHENOMENA
IN ACTIVATED SLUDGE

By

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THESIS

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BIOCHEMICAL STORAGE PHENOMENA

IN ACTIVATED SLUDGE

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The purpose of this research was to investigate the
biochemical composition of activated sludge, with particular reference
to storage material during both the period of activate assimilation
and the period of stabilization.

Poly-beta-hydroxybutyrate (PHB), a unique carbon-energy
storage material, was identified as a component of municipal treatment
plant activated sludge solids by the use of infrared analysis. More
positive identification was supplied when PHB was isolated from
laboratory activated sludge and characterized according to its melting
point, carbon-hydrogen-oxygen content and COD.

Glycogen, a carbohydrate storage material, was present in
very small amounts. Total cell carbohydrate material, on the other
hand, was stored to a much greater degree as judged by its rapid
synthesis and subsequent degradation. The influence of loading rate
(F/M ratio), COD/N ratio substrate on storage products was investigated.
The results indicated that the F/M ratio at which the activated sludge
is acclimated exerted an influence on the amount of storage material
that was synthesized. An optimum F/M ratio of 4.30 gm COD/(gm MLSS)(day)
provided cells that had the highest percentage of storage materials.

Cells grown in systems with greater or lesser F/M ratios did not
have as high a percentage of storage material. The COD/N ratio
had little effect on the percentage of storage compounds in the cell.
When fed glucose and yeast extract, a high COD/N ratio produced an
excess of biologically non-degradable carbohydrate material, in
addition to storage material. The type of substrate used had a
noticeable effect on the nature of the storage material. An activated
sludge unit, acclimated to a carbohydrate-ammonium ion substrate
accumulated neither carbohydrate nor PHB storage material. A source
of preformed amino acids, such as yeast extract, along with a
carbohydrate, was required in order to maintain a population from
the original municipal treatment plant seed that would synthesize PHB.

The ability of an actively stabilizing sludge to remove
substrate was investigated. It was found that as the cellular storage
products decreased during stabilization, the ability of the organisms
to remove substrate was greatly increased. When the storage products
reached a minimum level, the ability of the organisms to remove
substrate was at a maximum.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

The impetus for research in the area of biological treatment of wastewaters by activated sludge, or, for that matter, in any waste treatment process is apparent when one considers the worldwide interest in water pollution. Never before has there been such a great variety of people interested in the social and economic aspects of the discharge of foreign material into the streams and lakes of our country. Almost daily one may find evidence of this interest in nontechnical newspapers and magazines. Legislative activities have increased manyfold during the period from the first comprehensive-type legislation in the pollution control field (P.L. 845, 1948) to the present time. Both the state and the federal governments are expanding physically and fiscally to meet the current needs of research and development in water pollution control. Although the objectives of water pollution control are varied, one of the main areas of interest is the improvement of existing methods of waste treatment.

During the past fifty years, there has been a great deal of advancement in the theory and practice of treating wastes, especially by the activated sludge process which developed from the first laboratory experiments of Ardern and Lockett in 1913. This form of biological treatment has borne much of the burden of protecting the streams and rivers of the United States as well as those of many other nations. As judged by its rapid acceptance, activated sludge was recognized immediately as a practical method for waste treatment.

Initially, design criteria were based almost entirely on practical experience; but, as the diversity of wastes increased, the limits of the process apparently became more confining. It was then, through the efforts of laboratory researchers, that the theory of purification by bacteria began emerging and the building blocks of the process were carefully placed on a firm foundation. The natural co-existence of fundamental research and practical experience led to rapid improvement in the process and to wide applicability of the activated sludge process. This joint venture not only contributed greatly to the understanding of all biological waste treatment systems, but has also resulted in increased operational control.

Despite great technical advances there still remain many significant areas within the realm of biological treatment that require fundamental investigation. One such area is concerned with the influence of substrate storage by activated sludge organisms on the "condition" of the sludge when it is returned to the aeration tank.

It is generally believed that a portion of the assimilated organic matter removed from the waste stream during initial contact is stored by the microorganisms and that this stored material must be exhausted during prolonged aeration under starvation conditions before the sludge is in a suitable "condition" to be recycled. If the sludge is not so "conditioned" it will have a reduced capability for removing additional organic matter as well as reduced settling properties and thus will be described as poor. This process, in which activated sludge is aerated under starvation conditions, is a fundamental and

accepted principle in the operation of the activated sludge system
and many of its modifications.

The research reported herein deals with this very important
concept of storage in the activated sludge process by attempting to
delineate some of the factors which contribute to the synthesis of
storage materials. Thereafter, an attempt is made to evaluate the
influence of these storage products on the system. In order to
demonstrate the engineering significance of storage products, it was
considered necessary to actually identify a storage product in the
activated sludge from a municipal wastewater treatment plant.

II. THE CONCEPT OF STORAGE: A DEFINITION

The words "storage" or "storage products," as they relate to a microbial system, generally bring to mind certain identifications to the microbiologist while, on the other hand, the terms may have vastly different meanings to those persons interested in sanitary science. It is, therefore, necessary to define early in this writing these phrases that will be used over and over again.

The definition to be presented is one that more readily can be used in Sanitary Engineering than in microbiology. Nevertheless, the use of these words in the purer biological sense is also discussed in order for the reader to appreciate a later section which reviews literature on the storage functions in pure cultures.

To the microbiologist all cellular entities have a purpose for existence. Therefore, to label the entity is to predicate its function. This is true for the class of compounds known as "energy storage compounds" or its synonymous reference "carbon and energy storage compounds." However, the proposed functions of these compounds have not met with unanimous approval.

Wilkinson (1) has made a broad distinction between energy storage compounds that provide <u>both energy and carbon intermediates</u>, such as glycogen, starch, and poly-beta hydroxybutyric acid (PHB), and energy storage compounds that provide <u>only energy</u>, such as creatine phosphate.

To demonstrate the energy storage function, Wilkinson (1)

said the compound must:

1. "Accumulate under conditions when the supply of energy from exogenous sources is in excess of that required by the cell for growth.

2. "be used when the supply of energy from exogenous sources is insufficient for the optimal maintenance of the cell.

3. "be broken down to produce energy in a form utilizable by the cell...some purpose which gives the cell a biological advantage in the struggle for existence over those cells which do not have a comparable compound."

He warned that all three criteria must be met since there are various metabolic patterns in certain bacteria that could qualify some compounds as storage materials through the demonstration of one or two of the above conditions but fall short on fulfilling all three.

There are several reasons that have been advanced for the existence of storage compounds:

1. for cell growth in the absence of an external energy source.

2. for special phases of the division cycle.

3. for maintenance of cell integrity and viability.

4. for energy required in adaptation to a different medium.

5. for special mechanisms of survival.

Much has been written in support of one or more of the above proposals but in essence the ideas are representative of what constitutes the identification and function of carbon and energy storage compounds. Particular note is taken that no mention is made of the rate of synthesis or degradation of these compounds. This will be discussed later.

Let it suffice then to say that in the purer biological science, storage products may be described as compounds that are synthesized during exogenous substrate assimilation and are subsequently broken down in the absence of substrate for either their molecular constituents or energy or both.

The correlation between a storage function and bacterial endogenous metabolism is a conspicuous extension of the above discussion. Several recent reviews (2,3,4) have focused attention on energy storage compounds as the prime substrates for endogenous metabolism but they do not exclude the presence of other substrates which could be used for the same purpose such as ribonucleic acid (RNA), protein and free amino acid and peptide pools. Thus, there is developing the theory of bacterial endogenous metabolism in which a somewhat diverse array of compounds may act as energy storage material. This type of consideration provides a broader picture of the storage function in bacteria and consequently is more adaptable to sanitary science.

It is this more general view, which includes the role of endogenous metabolism, that should be used to define bacterial storage in an activated sludge system. The biological heterogeneity of activated sludge suggests the presence of some organisms that may call upon protein reserves while others use carbohydrate or lipid reserves during periods of starvation. Furthermore, a logical continuation would be to assume that other compounds exist which can also serve as substrates during endogenous metabolism.

In reviewing the literature pertaining to activated sludge, it appears that the word "storage" may have two meanings. Phelps (5) described the biochemical process of trickling filters and activated sludge as one of "adsorption, storage and oxidation." Rich (6) referred to storage in the following way: "That portion of the organics adsorbed initially that is not immediately oxidized or used in synthesis is stored in the activated sludge floc." Both these views undoubtedly refer to a physical holding that occurs within the zoogleal or slime matrix and not to intracellular substrate storage.

A second use of the word "storage" coincides more with the views expressed by microbiologists. One of the early references to the subject was contained in the series published by the U. S. Public Health Service, "Studies on Sewage Purification." In 1940, Ruchhoft et al. (7) found that "...in the metabolism of glucose by activated sludge only a small portion of the glucose that disappears is consumed in the respiratory process and a larger portion of the glucose is stored." Subsequently, Porges et al. (8) isolated the storage compound glycogen from activated sludge which had been acclimated to skim milk. Other investigators (9,10,11) have also implied an intracellular bacterial storage product in laboratory activated sludge units. The reference to storage in this present work will be solely in terms of the latter meaning, that is, a phenomenon associated with the metabolic activities of the microorganisms and not a physical holding action.

There is no rigid requirement that dictates whether storage products must be intracellular or extracellular; however, there is

considerable evidence that extracellular polysaccharides are rarely

degraded by either the microorganisms that produce them or other

microorganisms (12). Nevertheless, in view of the diversity of

organisms in the activated sludge process, it cannot be ruled out

that selection may encourage the growth of organisms capable of using

capsular material or slime as substrates for endogenous metabolism.

For the purpose of this work, the material identified as being stored

must be intracellular and be an integral part of the biomass.

Finally, in arriving at an acceptable and meaningful

description of storage products, it was considered necessary to

include a provision for the rate of synthesis and degradation. The

mechanism of removal of organic material in an activated sludge

system may be described as similar to the mechanism of stream

purification, except that the former takes place in a more confined

space and at a much higher rate. Since the element of time places a

restriction on the design criteria of activated sludge waste treatment

plants, it should also be represented in the concept of substrate

storage by activated sludge organisms. Therefore, to be classified as

a storage product, only consideration will be given to those materials

that can be synthesized and degraded in substantial quantities at a

rate in keeping with the design limits for the activated sludge process.

In view of the foregoing discussion, the following definition

for storage products is presented. It is to be used in conjunction

with the experimental work reported herein:

STORAGE PRODUCTS ARE THOSE COMPOUNDS ASSOCIATED
WITH THE INTEGRITY OF THE CELL AND WHICH UNDERGO
RAPID SYNTHESIS IN THE PRESENCE OF EXOGENOUS SUB-
STRATE AND RAPID DEGRADATION UPON EXHAUSTION OF
THE EXTERNAL FOOD SUPPLY.

III. PURPOSE AND SCOPE

In the activated sludge waste treatment process, degradable organic matter is removed from a waste stream by the action of aerobic microorganisms. The organisms are then separated from the waste stream and a relatively clean liquid remains to be discharged to a receiving body of water. During the initial contact of organisms and organic waste, the total amount of organic matter that is actually destroyed is represented by the amount of carbon dioxide that leaves the system. The remaining organic matter is assimilated into the various cellular constituents of the microorganisms thereby reflecting only a change in the structure of the original carbonaceous matter. The organisms must undergo additional treatment until as much of the assimilated matter is destroyed as is economically feasible. Generally, this is accomplished by keeping the organisms under aeration without the benefit of an additional food supply until the assimilated matter can be destroyed through the catabolic reactions of the microorganisms.

It would seem quite appropriate to investigate the intermediate steps involves in the conversion of the carbon in the wastewater to cellular carbon as well as the degradation of the cellular carbon under prolonged aeration. By following these transformations sufficient insight may be gained to increase the rates of total destruction of waste organic matter.

There is little information available as to the quantitative distribution of carbon into cellular constituents in the activated sludge

process and especially with regard to their rates of synthesis and degradation. Conceivably there may be one or two major arteries through which wastewater carbon is assimilated and degraded by bacteria. If so, the application of certain environmental controls may lead to the total destruction of greater quantities of organic matter and at a more rapid rate. A possible pathway for a major portion of wastewater carbon is into storage products since, for pure cultures, carbon storage products have been found to accumulate intracellularly upon exposure of the organism to substrate and are degraded in the absence of exogenous substrate (1,13).

The general purpose of this investigative effort was to focus attention on the composition of activated sludge during the removal of substrate and also during the stabilization period when there is no exogenous substrate available for the microorganisms. Particular attention was given to storage products (as defined in Chapter II) and their relative rates of synthesis and degradation as compared to other components of the sludge.

The three basic objectives were:

1. To qualitatively examine municipal and laboratory activated sludges for the presence of a known carbon-energy storage compound. In particular, the unique bacterial storage product, poly-beta-hydroxybutyric acid, was chosen because of its ease of identification through infrared analysis and because it has been shown to be important as a storage product in cells grown in pure culture.

2. To determine the influence of certain accepted process control parameters on the synthesis of storage products. These were: food to microorganism ratio (F/M), COD/N ratio, and the influence of substrate composition.

3. To determine the influence of storage products on the operation of the activated sludge process. Two of the main criteria of operation are the removal of substrate and the settling characteristics of the sludge. Both of these parameters were investigated.

IV. LITERATURE REVIEW

"One great use of a review, indeed, is to make
men wise in ten pages, who have no appetite for
a hundred pages; to condense nourishment, to
work with pulp and essence, and to guard the
stomach from idle burden and unmeaning bulk."

Sidney Smith
Edinburgh Review, 1825

A. Development of the Substrate Storage Concept in Activated Sludge

In the fifty some years since Ardern and Lockett (14) first

published results of their experiments on what was to become known as

the activated sludge process, research in the area of biological waste

treatment has expanded at a rate comparable to the development of the

airplane. This expansion was accompanied by a voluminous increase in

the published literature so that after fifty years a comprehensive

critical review of the literature becomes a full-time single effort

in itself. Consequently, the purpose of the following review is to

cover only those past efforts that are in keeping with the purpose

and scope of this investigation. A brief history of the fundamental

principles of activated sludge waste treatment has been considered to

complement the remaining portions of the literature survey.

A number of early investigators (15), from 1918 to 1930,

reported on the rapid removal of organic colloids when sewage was

brought in contact with filter slimes or activated sludge. Because

of the existing theory that bacterial purification was a slow process

that required days instead of hours, these observations led many to

believe that microorganisms, per se, were not the major factor in the primary clarification of sewage. It was generally believed that the theory of sewage treatment by activated sludge involved a rapid physical adsorption of the organic material by the sludge, followed by bacterial oxidation of the adsorbed matter (16).

Considerable controversy existed regarding the rapid initial "clarification" step. Among the theories presented were: a) mutual coagulation of the sewage colloids through charge neutralization b) agglomeration of positively charged bacteria and negatively charged sewage colloids and c) agglomeration of weakly charged colloids with strongly charged bacteria of the same sign (15). These theories fitted the explanation for rapid "clarification" but some of them seemed to defy the laws of physics. Theriault (17) believed the adsorbent principle to be a biozeolitic mechanism when he identified a base-exchange substance in activated sludge. The biological theories of clarification, such as bioprecipitation by Buswell (18) and the enzymatic theory of Parsons (19) did not meet with much approval because of the fact that the rates of clarification were not consistent with the then current views on bacterial oxidation rates. However, in 1936 McNamee (16) presented data to show that sewage matter could be oxidized much more rapidly than had generally been realized. The following year, Butterfield et al. (20) studied the biochemical oxidation of sterile sewage and synthetic sewage by pure cultures isolated from treatment plant activated sludge. They conclusively established that oxidation velocity constants in the activated sludge process were, indeed, much

greater than the constant for natural biochemical oxidation as observed for streams (21). This finding constituted a major advance in the fundamental principles of aerobic biological treatment. Although the mechanisms involved in the purification of polluted waters, whether they are natural streams or sewage plant influents, were basically similar, the rates of purification were different: a slow rate for natural purification and a rapid rate as found in the activated sludge process.

Thereafter, the intensity of investigations increased and answers were forthcoming as to the effect of initial numbers of bacteria on BOD removal (22) and on the influence of BOD loading on the system (23). In 1939, Ruchhoft et al. (24) demonstrated and measured, for the first time, the portion of the total BOD of a waste that was oxidized. The remainder was said to be adsorbed or synthesized. Eighty to 90 per cent of the total carbonaceous BOD was removed in 5 hours of aeration with activated sludge. Thirty to 60 per cent of the BOD removed was oxidized and the remainder accounted for the net adsorption and synthesis.

In 1940, Ruchhoft et al. (7), using glucose and municipal treatment plant activated sludge, proposed that a large portion of the glucose removed from solution was stored by the organisms and only a small portion was respired. As a consequence, they felt that assimilation and not dissimilation should be emphasized in the activated sludge process. In referring to "stored" material, they were actually thinking of intracellular carbohydrates inasmuch as they

referred to the work of Winzler and Baumberger (25) who had
investigated glycogen synthesis in yeasts. This was, it is believed,
the first time the storage phenomenon had been mentioned in the
sanitary engineering literature.

It wasn't until 1955 that the phenomenon of storage was
again mentioned. The excellent series of papers by Hoover and Porges
(26,27,28,29,30,31,32) on the assimilation of dairy wastes by activated
sludge, culminated in 1955 with a report (8) in which they proposed
three distinct phases associated with the purification of soluble
substrate: oxidation, synthesis, and storage. They defined
purification as a combination of the three and measured it by the
removal of COD from supernatant liquor. Since their studies are the
only ones in which storage products were directly measured in a
heterogeneous bacterial system, their work will be reviewed in
somewhat more detail than other references.

The growth unit used by Hoover and Porges consisted of a
20-liter fermentor seeded with activated sludge from a dairy waste
treatment plant and was continuously fed skim milk at the rate of
1 liter per hour (26). Their source of activated sludge organisms
for all experiments, including those using the Warburg respirometer,
were obtained from this unit.

From an elemental analysis of the sludge after 48 hours of
endogenous respiration (29,31) they developed an empirical formula for
activated sludge, $C_5H_7NO_2$, which is conveniently referred to in this
review as the "nominal cell." The percentage of elemental constituents

in this formula is compared with other published data in Table 1. The
somewhat higher oxygen content in the activated sludge organism could
possibly be due to the environmental conditions under which the cells
were cultivated. While the <u>Escherichia</u> <u>coli</u> cells were obtained
during the log phase of growth, the activated sludge organisms were
obtained from laboratory fill and draw systems in which the daily
exposure to long periods of endogenous metabolism could conceivably
produce organisms whose cellular constituents were highly oxidized.

Using this formula, Hoover and Porges (29) were able to
write a balanced equation for the synthesis of cellular material from
skim milk, as follows:

$$8(CH_2O) + C_8H_{12}N_2O_3 + 6\ O_2 = 2\ C_5H_7NO_2 + 6\ CO_2 + 7\ H_2O\ .\ .\ .\ 1$$

Lactose   Casein   Sludge
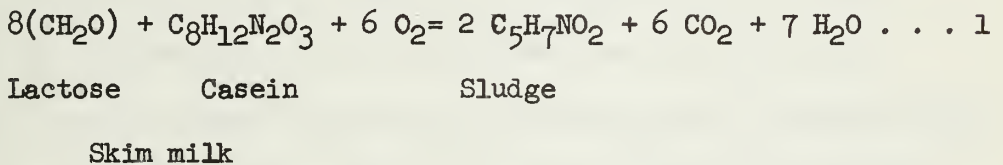
      Skim milk

In this equation 16 carbon atoms of skim milk are used to produce 10
carbon atoms of sludge and 6 carbon atoms of carbon dioxide. Since 6
moles of oxygen would be used to produce 6 moles of carbon dioxide.
Since 6 moles of oxygen would be used to produce 6 moles of carbon
dioxide, the validity of the equation could be conveniently determined
by measuring the oxygen uptake of the sludge during oxidative
assimilation of skim milk. For every mg/l of oxygen uptake there
should be 16/6 or 2.67 mg/l of skim milk COD removed from solution;
that is, if the equation were to hold true.

TABLE 1

COMPARISON OF THE ELEMENTAL COMPOSITION OF A BACTERIAL CELL
AND ACTIVATED SLUDGE (ASH FREE BASIS)

Element	E. coli	Per Cent of Total Weight			
		Activated Sludge Grown on			
		Skim Milk*	Ind. Wastes	Acetic Acid	
				With Nitrogen	Without Nitrogen
	(33)	(29)	(34)	(35)	(35)
Carbon	52.6	51.7	53.5	48.2	51.7
Hydrogen	10.5	6.3	7.0	6.9	7.2
Oxygen	21.0	29.5	30.6	36.8	38.6
Nitrogen	15.8	12.4	8.9	8.0	2.4

*Hoover and Porges' Sludge

Numbers in parenthesis are reference numbers.

The validity of the equation was determined by actually
measuring the COD removal in a jar experiment and simultaneously
predicting the COD removal from oxygen utilization data obtained using
a Warburg Respirometer. Almost 80 per cent of the substrate COD (as
measured) was removed from solution in one hour, whereas, in the Warburg
flask only 50 per cent of the COD was satisfied (as calculated from
equation 1 and the amount of oxygen utilized). The remaining 30 per
cent of the COD that was removed, but not oxidized, was considered to
be stored intracellularly.

A further study was conducted (8) in which the time course of
purification, oxidation, synthesis, and storage was studied over a
20-hour period. The analyses performed in the experiment were CO_2
evolution and COD of the supernatant. Table 2 is a composite of data

TABLE 2

DISTRIBUTION OF SUBSTRATE COD DURING OXIDATIVE ASSIMILATION
OF SKIM MILK BY ACTIVATED SLUDGE.   20° C*

Time	Per Cent of COD Removed			
(hrs)	Purification[a]	Respiration[b]	Synthesis[c]	Storage[d]
1	67.9	5.3	8.9	53.7
2	73.4	8.3	13.9	51.2
3	89.0	11.0	18.4	59.6
4	92.9	13.1	21.8	58.0
20	95.0	24.9	41.5	28.6

[a]Purification: COD actually <u>measured</u> as the COD removed from supernatant.

[b]Respiration: Actually <u>measured</u> as CO_2 evolved.

[c]Synthesis: Calculated by converting CO_2 evolved to O_2 uptake which in turn was multiplied by 1.67 in accordance with equation 1. It represents the amount of substrate COD removed that goes into the synthesis of a nominal cell.

[d]Storage: <u>Calculated</u> as the difference between the actual COD removed and the COD removed for purpose of oxidation plus synthesis of the nominal cell. It represents the amount of substrate COD removed that goes into the synthesis of material other than that required for the nominal cell. Storage = 1 - (2+3).

* Data obtained from reference 8.

obtained from several tables presented by Porges et al. (8). Assuming

there was no storage in the cell at time zero, the data show and

extremely rapid rate of storage during the first hour with little

change over the next three hours. Complete degradation of the storage

material was not observed, since 28.6 per cent of the substrate removed

from solution was still in the form of storage material at the end of

20 hours. However, in a similar experiment conducted at 30° C, they

found that after 20 hours none of the substrate removed from solution

could be accounted for as storage material. Two other experiments con-

ducted at 2° C and 10° C led them to conclude that the storage ability

of the organisms was temperature dependent. They definitely implicated

glycogen as the storage product by isolating the material from large

samples of mixed liquor. Glycogen determinations on mixed liquor

harvested after an aeration period of 5 hours at 30° C showed that

19.3 per cent of the solids weight contained this carbon-energy storage

compound. After 24 hours this value decreased to 8.3 per cent. The

finding of glycogen was the first direct evidence of the presence of

a true storage material in a heterogeneous population such as activated

sludge.

Others have implied the presence of energy-storage products

in activated sludge systems. Gaudy and Engelbrecht (9), using glucose

acclimated sludge, found almost twice as much sludge carbohydrates

formed in a respiring system (nitrogen free) as in a growth system

(with nitrogen). They implied that much of the carbohydrate was

intracellular storage product which was subsequently used for protein

synthesis.

McWhorter and Heukelekian (10) also implied the conversion of glucose into storage products. Using activated sludge seed, as well as sewage seed, they found the amount of oxygen required to oxidize 1000 mg/l glucose (as COD) was dependent on the initial concentration of seed organisms. As the seed concentration increased, the per cent of theoretical oxygen demand that was actually exerted decreased from 27.5 per cent to 21.0 per cent. This indicated that the higher F/M ratio system removed a greater portion of the glucose without oxidation. Since cell yields were independent of seed concentrations, the glucose removed without oxidation was assumed to be channeled into storage material. Their method of data presentation, as McKinney (36) pointed out, was a "...most significant contribution...," and is worthy of comment. They plotted all dependent variables against the per cent of theoretical oxygen uptake, thus doing away with rate considerations. In this type of approach to data presentation, the presence or absence of lag periods during growth does not influence data analysis. Furthermore, the procedure is not limited to substrates of known chemical formula, since theoretical formulas can be obtained for any organic matter by the use of elemental analysis.

In a ten-day extended aeration experiment designed to investigate endogenous metabolism, McWhorter and Heukelekian (10) measured sludge mass, oxygen uptake, cellular (total organic) nitrogen and ammonia nitrogen in the supernatant. Since all their data were on a "per cent of theoretical oxygen uptake" basis, it might be well to

point out, in the interest of clarity, that this term can be generally thought of as equivalent to time. Figure 1 is a reproduction of their data. When all the substrate COD had been removed (at 30 per cent of theoretical oxygen demand), the cell mass was at a maximum but the cell nitrogen continued to increase until 35 per cent of the theoretical oxygen demand had been exerted. Thereafter, the cell nitrogen decreased and the ammonia nitrogen in solution increased. They attributed the decrease in cell mass and increase in cell nitrogen (phase A) to the utilization of stored material, "probably carbonaceous matter." Phase B had an oxygen to ammonia ratio of 5.9/1 which is close to that for the complete oxidation of protein (37), thus indicating that a majority of the endogenous substrate in this phase could have been protein. This was further substantiated by the 1.4/1 ratio of oxygen uptake to cell weight loss in this region. Protein has a ratio of 1.43/1. In Phase C, the oxygen/ammonia ratio was nearer 20/1, indicating that nonprotein material was serving as the major source for endogenous substrate. These findings are in general agreement with those of Ribbons and Dawes (3) on the endogenous metabolism of Sarcina lutea.

The use of non-carbonaceous material as substrate for endogenous metabolism has been indicated by several investigators. Besides the pure culture studies of Ribbons and Dawes (3), in which they found the amino acid pool to be depleted concurrently with a loss in glycogen, there have been studies in which activated sludge organisms were shown to deplete non-carbohydrate material.

Van Gils (38) found that endogenous respiration took place
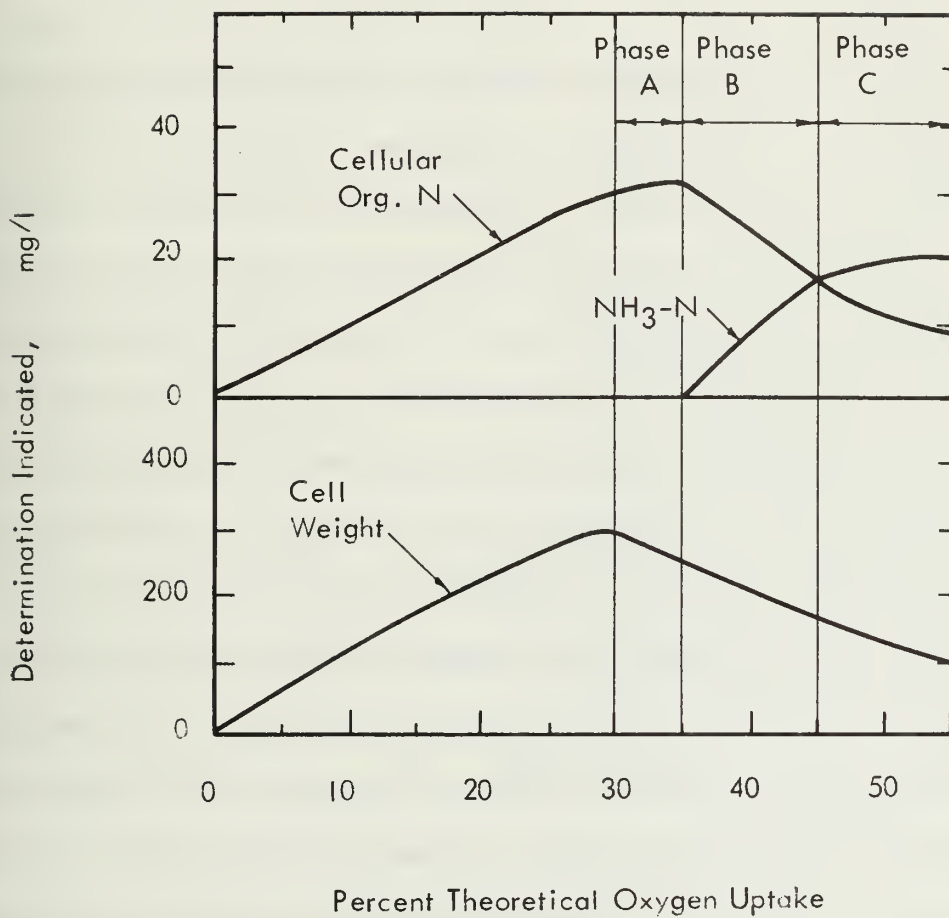
FIGURE 1   CHANGES IN CELL NITROGEN, RELEASED AMMONIA AND
CELL WEIGHT FOR EXTENDED PERIODS OF AERATION. DATA
FROM McWHORTER AND HEUKELEKIAN (10)

at the expense of an unidentified fraction of the sludge. When he measured sludge carbohydrates (anthrone) and protein (Kjeldahl nitrogen) they decreased only slightly, while the unidentified fraction, as determined by solids difference, accounted for the major portion of the loss in sludge weight. He concluded that the formation of lipid storage products was of minor importance and that the major substrate for endogenous metabolism was neither carbohydrate nor protein.

In a study by Washington and Symons (39), using glucose, acetate, and glycine as substrates for three laboratory activated sludge systems, they found that all components of the sludge (carbohydrate, protein and fat) were degraded during a prolonged period of starvation. Protein, they said, was the primary source of energy and assimilation for the endogenously respiring organisms regardless of the substrates to which they were acclimated. The assimilation activity during endogenous metabolism was directed toward synthesis of biologically inert material which they reasoned was cell capsule and external slime. Their systems were considerably biased, however, since there was no wasting of sludge and the COD/N ratio of the feed was 133. Under such circumstances, with a nitrogen deficient feed and growth in an almost biologically closed system, the build-up of inert material is inevitable. It is difficult to understand how they ever reached a solids equilibrium level as they claimed.

Symons and McKinney (35) obtained similar results in non-wasting batch activated sludge systems that were operated at nitrogen levels varying from 0 to 50 mg/l. After 35 days of operation

each sludge was stained with Alcian Blue and the relative amounts of
stainable material noted as a measure of the extracellular
polysaccharide content. They also measured volatile solids and cell
nitrogen at frequent intervals. The general trend of their data showed
an increasing solids build-up, a decreasing nitrogen content in the
sludge and a build-up of polysaccharide content with time. From these
data they indicated a difference in the type of carbohydrates
synthesized as well as the mechanism for synthesis as compared to that
proposed by Porges (40). Whereas, Porges had indicated the synthesis
and subsequent degradation of intracellularly stored polysaccharides
like glycogen, Symons and McKinney believed the polysaccharide
accumulation took place extracellularly to form capsular material.

The seemingly antithetic mechanisms postulated for
polysaccharide accumulation by activated sludge organisms are in
reality probably quite complementary. There is no reason to believe
that both synthetic mechanisms cannot exist simultaneously during
substrate metabolism. If some quantitative definitions were applied to
these results, it might be expected that both intracellular storage
products as well as extracellular capsules are synthesized during
oxidative assimilation and subsequent starvation stages of the
activated sludge process.

The use of non-protein nitrogenous matter as substrate for
endogenous metabolism has been shown in pure culture studies (3) as
well as in activated sludge studies (10) but little attention has been
given to whether this material could be of an extracellular nature.

Not all extracellular polysaccharides are of the pure carbohydrate class of compound. Many contain nitrogen and, in particular, hexosamines. This compound has been identified in pure culture isolates of Zooglea ramigera (41) and from wastewater treatment plant activated sludge (41) as well as from laboratory activated sludge (33). The possibility is perhaps remote but there are some organisms that can break down extracellular capsular material. Bacillus subtilis, an organism that has been found in activated sludge (42), produces a polyglutamic acid capsule that it can readily hydrolyze (43). Similarly, a Flavobacterium sp. has also been identified that can synthesize and then hydrolyze the glutamyl bond of the same polypeptide (44). The latter genera has been frequently isolated from activated sludge (45,46,47). The possibility therefore exists that extracellular non-protein nitrogenous matter can act as a substrate for endogenous metabolism.

Besides glycogen there are other true carbon-energy storage compounds. One that has been frequently studied using pure cultures is the lipid, poly-beta-hydroxybutyrate. It is only within the past two years that this compound has been mentioned in regard to its possible role in the activated sludge process. Dias and Bhat (48) found that during a survey of the bacterial flora of activated sludge, 52 per cent of the dominant bacteria isolated by plating sludge on plates of sewage solidified with agar contained sudanophilic granules, typical of PHB. Twelve strains belonging to the genera Zooglea and Comamonas were grown in a peptone-glycerol-succinate medium, harvested, and extracted with boiling chloroform. The extract was precipitated in

ethyl ether, dried and then subjected to physical and chemical analysis (49). Melting point determinations, solubility in various solvents and elemental analysis all indicated that the isolated material was PHB.

Crabtree et al. (50) isolated PHB from Z. ramigera by acid hydrolysis, hot chloroform extraction and precipitation in ether. Physical and chemical analysis included: melting point, solubility and infrared analysis. All results indicated that the isolated material was PHB. In addition, infrared analysis of washed activated sludge from a wastewater treatment plant produced a strong absorption similar to the isolated PHB as well as a commercial preparation of PHB. When the organism was incubated with a limiting carbon source, no PHB was formed and no flocculation took place. On the other hand, in the presence of excess substrate, large quantities of PHB were formed and flocculation was evident. Since capsule or "gum" polysaccharides were not demonstrable on the cells, it was assumed that flocculation was closely associated with the intracellular accumulation of PHB. Furthermore, deflocculation was observed to coincide with the depletion of PHB in a washed suspension of the organisms. Their results implied that PHB accumulation was associated with the mechanism of flocculation.

The base composition of activated sludge in terms of protein, carbohydrate and fat has been reported by several investigators (38,39, 51, 52, 53). It is somewhat difficult to compare their values because of differences in loading, acclimation, substrate used and analytical methods. Nevertheless, in Table 3 is presented selected data on the

TABLE 3

BASIC PROTEIN, CARBOHYDRATE, AND FAT COMPOSITION OF ACTIVATED SLUDGE

Type of Operation	Substrate Acclimation	Per Cent of Solids			Reference
		Protein	Carbohydrate	Fat	
Batch (NW)	Sodium Acetate	40.3 (N)	53.3 (H)	6.1	39
Batch (NW)	Glucose	54.0 (N)	39.8 (H)	6.2	39
Batch (NW)	Glycine	67.0 (N)	26.6 (H)	6.4	39
Flow Thru (NW)	Sodium Acetate	59.0 (N)	34.9 (H)	6.1	39
Flow Thru (NW)	Glucose	52.0 (N)	41.0 (H)	7.0	39
Flow Thru (NW)	Glycine	69.1 (N)	24.5 (H)	6.4	39
Flow Thru (R)	Glucose	14.2 (B)	17.5 (A)		51
Flow Thru (R)	Sorbitol	21.0 (B)	29.0 (A)		51
Batch (W)	Glucose	39.0 (N)	23.2 (A)	15.0	38
Flow Thru (C)	Sorbitol	54.0 (B)	30.0 (A)		52
Batch (Log)	Glucose	40.0 (B)	20.0 (A)		53

(NW): Non-wasting system. All sludge retained.

(R): Some sludge recycled.

(W): 20 per cent sludge wasted per day.

(C): Operated on the Chemostat principle. No recycling of sludge.

(Log): Batch type organisms acclimated to a wasting procedure and then
       subjected to true log growth.

(N): Protein determined from Kjeldahl nitrogen values.

(B): Protein determined by the Biuret test.

(H): Carbohydrates determined by acid hydrolysis followed by Somogyi
      procedure for reducing sugars.

(A): Carbohydrates determined by the Anthrone test.

basic composition of activated sludges grown on various substrates. In
cases where the composition changed during the metabolism of a substrate,
the zero hour value was used. From the information presented it appears
that nitrogen analysis is not the best method for determining protein.
The higher protein content found in cells using this test is probably
due to the fact that a portion of the nitrogenous material in activated
sludge is in a non-protein form. It appears that sludge grown in a
chemostat without recirculation and sludge grown in a non-wasting
system has a higher basic protein content than systems operated on a
fill and draw basis. The carbohydrate content of sludge varies consider-
ably depending on the substrate used as well as the conditions under
which it was grown.

Considerable controversy exists concerning the qualitative
and quantitative nature of storage products in the activated sludge
system. Many of these differences seem to be a result of the cultural
conditions of study; the substrate used, the analytical test
procedures and the type of system operation. Much work needs to be
done on the role of storage in the activated sludge process.

With the study of activated sludge dealing more and more
with the cytological aspects of the bacteria, it would behoove the
research scientist to develop a laboratory model that more closely
approximates the actual treatment plant and thus provide a bacterial
model with similar metabolic activities to those under field conditions.

B. Storage Products in Pure Cultures

The concept of storage material in pure cultures of bacteria has been reviewed in Chapter II along with their function and the requisites for demonstrating their existence. In the following pages, storage products will be discussed as to their occurrence in various organisms, the substrates used for synthesis and the environmental conditions necessary for the synthesis and degradation of two of the more important storage products.

Many compounds have been assumed to act as storage materials in bacteria. Those most commonly implicated as specialized carbon and energy reserves are the lipid, poly-beta-hydroxybutyrate, and the polysaccharide, glycogen. Although in some instances both have been demonstrated in one species (54,55), namely Rhodospirillum rubrum and B. megaterium, storage compounds are generally identified with particular species.

Although the general metabolism of these polymers has been studied, little attention has been given to correlating their presence in relation to endogenous metabolism. Dawes and Ribbons (2) have presented an excellent review of endogenous metabolism in which they have included substrates other than the aforementioned carbon-energy storage compounds. Another excellent review was presented in a series of papers from a conference entitled Endogenous Metabolism With Special Reference to Bacteria which was held by the New York Academy of Science (56).

1. Poly-beta-hydroxybutyrate

In 1927 Lemoigne (57) demonstrated that a substance having the empirical formula $(C_4H_6O_2)_n$ could be extracted from cells of B. megaterium. He was able to show that the material was a polymer of beta-hydroxybutyric acid. Later, Lemoigne et al. (58) were able to correlate the occurrence of refractile lipid inclusions in a number of Bacillus cultures with the presence of appreciable amounts of cellular PHB.

Besides the occurrence of this polyester in a number of Bacillus species, it has been found in a variety of other bacteria. A partial list of bacteria (according to genera) in which PHB has been identified is given in Table 4. Included in the list are bacteria that have been isolated from activated sludge. It is quite interesting to note the presence of Sphaerotilus natans and Z. ramigera, two widely publicized organisms found in activated sludge.

Quantitatively, the amount of this polymer varies considerably and is dependent on the species, substrate and cultural conditions. Up to 50 per cent of the dry weight of the cell has been reported. The polymer is unique to bacteria and has been studied extensively during recent years.

Poly-beta-hydroxybutyrate appears as refractile granules inside the cell. They stain an intense blue-black with Sudan Black. Isolation of these granules by Williamson and Wilkinson (61) has shown that they are composed of about 90 per cent PHB and 10 per cent of some other lipids. The isolated material is generally white but has been

described as a "...thin greyish translucent membrane somewhat reminiscent of plastic sheeting..." (61). During acetone or ether precipitation, white translucent flakes form (50). The melting point varies considerably: 160 to 164° C (61), 156 to 172° C (49). Numerous chemical properties of the polymer have been described by Kepes and Leoel (62) and Williamson and Wilkinson (61).

TABLE 4

BACTERIA THAT HAVE BEEN FOUND TO CONTAIN POLY-BETA-HYDROXYBUTYRATE
AND THAT HAVE BEEN ISOLATED FROM ACTIVATED SLUDGE

Genera	References	
	Pure Culture	Activated Sludge
Bacillus	58, 59, 60, 61, 62	42, 46, 47, 48
Comamonas	48	48
Micrococcus	63, 64	46, 47, 48
Nocardia	65	47
Pseudomonas	66, 67	45, 47, 48
Spirillum	68, 69	48
Sphaerotilus[a]	68, 69	63
Zooglea[b]	48, 50	41, 47, 48, 50

[a]All references were for Sphaerotilus natans

[b]All references were for Zooglea ramigera

One of the most striking properties of PHB is its specific absorption in the infrared region. A number of investigators (50,64,65, 70,71,72) have found a strong absorption peak between 5.7 and 5.8 microns for cells that contain the polymer as well as for the isolated

PHB itself.

The effect of cultural conditions on polymer production in B. megaterium and B. cereus has been studied by MacRae and Wilkinson (59). Suitable substrates for polymer synthesis were: glucose, pyruvate, or beta-hydroxybrtyrate. Acetate, although alone unable to induce synthesis, greatly enhanced polymer formation in the presence of the other substrates listed. Exhaustion of the inorganic nitrogen source in the presence of excess glucose increased the accumulation of PHB considerably as compared to when glucose was limiting. In the absence of an external carbon and energy source, aerobic degradation of PHB occurred rapidly to carbon dioxide and water. The optimum pH for both breakdown and synthesis was found to be about 7.5. However, Nakata (73) has shown polymer accumulation to be inhibited at pH 7.5 and found the optimum to be at pH 6.4 for B. cereus.

Doudoroff and Stanier (67) investigated the synthesis and degradation of PHB during oxidative assimilation by Pseudomonas saccharophila and during the photoassimilation by R. rubrum. They found that with most substrates, 60 to 90 per cent of the assimilated carbon was accumulated initially as PHB. The fate of the accumulated PHB was obtained with washed suspensions in the presence of nitrogen. Although both organisms used the stored polymer, R. rubrum exhibited a more rapid rate of degradation and, in addition, redistributed much of the material into other cellular components. Degradation by P. saccharophila was reported to be at a slow rate and the redistribution on polymer carbon could not be demonstrated.

Other studies have shown bacterial degradation of PHB to be a very slow process. Sierra and Gibbons (63), while aerating a washed suspension of Micrococcus denitrificans, found the PHB content to be reduced from 55 per cent of the dry weight to 29 per cent in 127 hours. Cells initially containing 10 per cent PHB were starved for three hours during which time the PHB content decreased 50 per cent but no change in other cellular constituents was noticed. The extremely long periods of slow degradation were attributed to an inhibition produced by products of metabolism or lysis, since the inhibition could be removed by washing the cells.

2. Glycogen

The polyglucose material, glycogen, has been observed in a number of bacteria including: E. coli (74,75), Aerobacter aerogenes (76), B. cereus (69), B. megaterium (55), Arthrobacter (69) and Sarcina lutea (3).

As much as 75 per cent of the dry weight of Arthrobacter sp. has been found to be a glycogen-like polysaccharide (69). When washed suspensions of these cells were starved for four days only 40 per cent of their carbohydrate was utilized thus indicating that not all cell carbohydrate can be used as a substrate for endogenous metabolism.

Ribbons and Dawes (3) have shown that in E. coli, the possession of glycogen by the cells prevented a net degradation of nitrogenous material. Ammonia was released only after the glycogen had been oxidized. The depletion of up to 40 per cent (dry weight) of the

glycogen functioned as a reserve of carbon skeletons and not energy because of the transfer of carbon atoms from glycogen to other cellular constituents.

In S. lutea, however, the same authors (3) found that the oxidation of this carbohydrate occurred simultaneously with the depletion of the amino acid pool. The free ammonia released during starvation conditions, indicated that both glycogen and the amino acid pool were being used as endogenous substrates. The above results were for cells grown on glucose-peptone. When only peptone was used as the substrate, the amino acid and peptide pools were depleted during starvation but the carbohydrate and lipid fractions remained unchanged.

Strange et al. (76) found A. aerogenes cells, grown in a tryptone-glucose medium contained 5 to 20 per cent carbohydrates of which a high per cent was glycogen. During endogenous respiration, the glycogen was depleted at a much slower rate than that observed for E. coli cells (3). Ammonia was also released during starvation. In neither a tryptic meat broth nor a mannitol-ammonium ion medium were there significant amounts of glycogen found in the cell.

V. METHODS AND MATERIALS

A. Experimental Equipment

1. Cultivation in Fill and Draw Activated Sludge Units

All fill and draw laboratory activated sludge units were operated in 2-liter rectangular aeration tanks made from plexiglass (Figure 2). The stock unit, seeded with activated sludge from the waste treatment plant operated by the Urbana-Champaign Sanitary District was operated on a 24-hour fill and draw basis as follows: after 23.5 hours of aeration, the sides of the unit were cleaned of solids and make-up water added to compensate for evaporation. Fifty per cent of the mixed liquor was wasted and the aeration discontinued for one-half hour while the sludge settled. A maximum amount of supernatant liquor was drawn off and the unit refilled to the one-liter volume with tap water. The nutrients and substrates were then added. Finally the unit was brought up to the initial 2-liter volume with tap water and aeration resumed.

Experiments, which were designed to investigate the response of activated sludge to a substrate other than that used in the stock unit, necessitated the acclimation of sludge to a new substrate. The procedure was as follows: 10 ml of the stock mixed liquor was added to 2-liters of the new substrate solution and the contents aerated for 24 hours. The procedure was repeated using a 10-ml inoculum of the newly-formed mixed liquor. Thereafter, the wasting and feeding schedule, as described above, was maintained, but settling and the
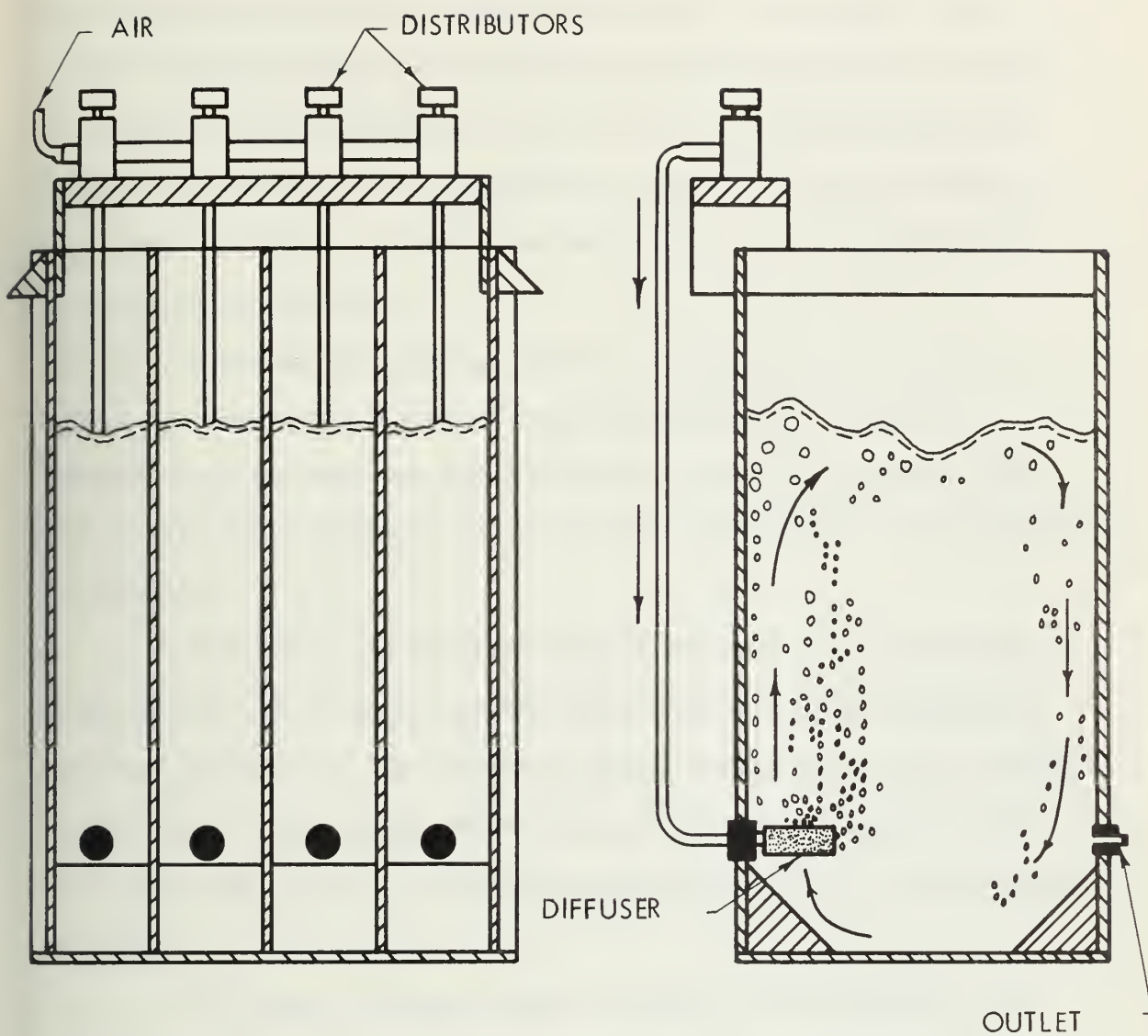
FIGURE 2 FILL AND DRAW LABORATORY ACTIVATED SLUDGE UNITS

discarding of supernatant was not begun until the sludge showed good settling characteristics. The acclimation of a new system in this manner is closely allied to an enrichment culture technique and would encourage only those organisms best able to use the substrate under study. All new units were acclimated a minimum of ten days before an experiment was made. By this time the sludge usually possessed good settling characteristics.

There was no external control of temperature, hence, the operating temperature varied as the room temperature (22-28° C). On several occasions the room temperature rose to 35° C; however, there was no noticeable effect on the condition of the sludge or the operation of the unit.

The stock unit operated without interruption for more than eight months during which time the major portion of the experimental work was carried out. To insure uniformity from week to week, periodic measurements of the sludge volume index (SVI) and pH were made. The SVI ranged from 35 to 45 while the pH never varied beyond the range of 6.8 to 7.4.

The stock activated sludge, developed and maintained on a glucose-yeast extract substrate, was found to be absolutely free from bulking problems. During the data collection period of this research, there were a number of projects in the Sanitary Engineering Laboratory dealing with the activated sludge process. Several of the laboratory units that did not employ a substrate with an organic nitrogen source experienced some difficulty with bulking from time to time. This

contrast provided interesting tangential reflections on the causes of laboratory sludge bulking.

Poly-beta-hydroxybutyrate is a unique storage compound which is found only in certain bacteria. Primary attention was, therefore, given to maintaining as heterogeneous a population as would be found in the initial seed from the Urbana-Champaign waste treatment plant. For this reason glucose, yeast extract, and ammonium sulfate were chosen as the carbon and nitrogen sources for growth. It was assumed that glucose, which is as close to a universal carbon source for microorganisms as any other single carbon compound, served to maintain the presence of the vast majority of the microorganisms from the initial seed. For those organisms that required preformed amino acids and protein in order to grow, yeast extract was added. All too frequently in the past, investigators have used the ammonium ion as the only source of nitrogen. This practice results in the selection of certain organisms to the exclusion of many others that may be found in waste treatment systems. Since amino acids and proteins are found in sewage (77,78,79,80) this particular nitrogen source should provide for a more realistic biota for laboratory studies involving biological waste treatment.

The stock unit was fed in such a way that the final concentration of nutrients, just after feeding, were as shown in Table 5. Trace minerals were provided in tap water. The pH of the above solution was 7.2. Glucose had a measured COD of 1.045 gm/gm of glucose while yeast extract had a COD of 0.998 gm/gm of yeast extract and a total nitrogen content of 0.075 gm/gm of yeast extract. Thus the COD of the

feed solution was 2590 mg/l and the total nitrogen content, from both organic and inorganic sources, was 257 mg/l. The COD/N ratio was, therefore, 10.1.

TABLE 5

CONCENTRATION OF SUBSTRATE AND INORGANIC SALTS USED
IN THE STOCK FILL AND DRAW LABORATORY UNIT

	mg/l
Glucose	2,000
Yeast Extract	500
Ammonium Sulfate	1,000
Magnesium Sulfate (7 waters)	50
Manganous Sulfate (1 water)	2.5
Sodium Chloride	1.0
Calcium Chloride (2 waters)	3.3
Ferric Chloride (6 waters)	0.5
Potassium Phosphate (Dibasic)	1,242
Potassium Phosphate (Monobasic)	221

2. Cultivation in a Continuous Flow Activated Sludge Unit

The continuous flow activated sludge unit is shown in Figure 3. It consisted of a 4-liter reservoir bottle from which feed was pumped to a 500-ml aeration vessel by means of an electrolytic pump. The mixed liquor flowed from the aeration vessel into a 4-liter stabilization vessel and thence to an effluent collector. There was no provision for settling or the return of sludge.

The electrolytic pump was similar to the one described by Symons (82). The control system and gas generator are shown in Figure 4. The gas generator, made from a 38 x 200mm culture tube, provided the necessary gas pressure to force substrate from the reservoir to the
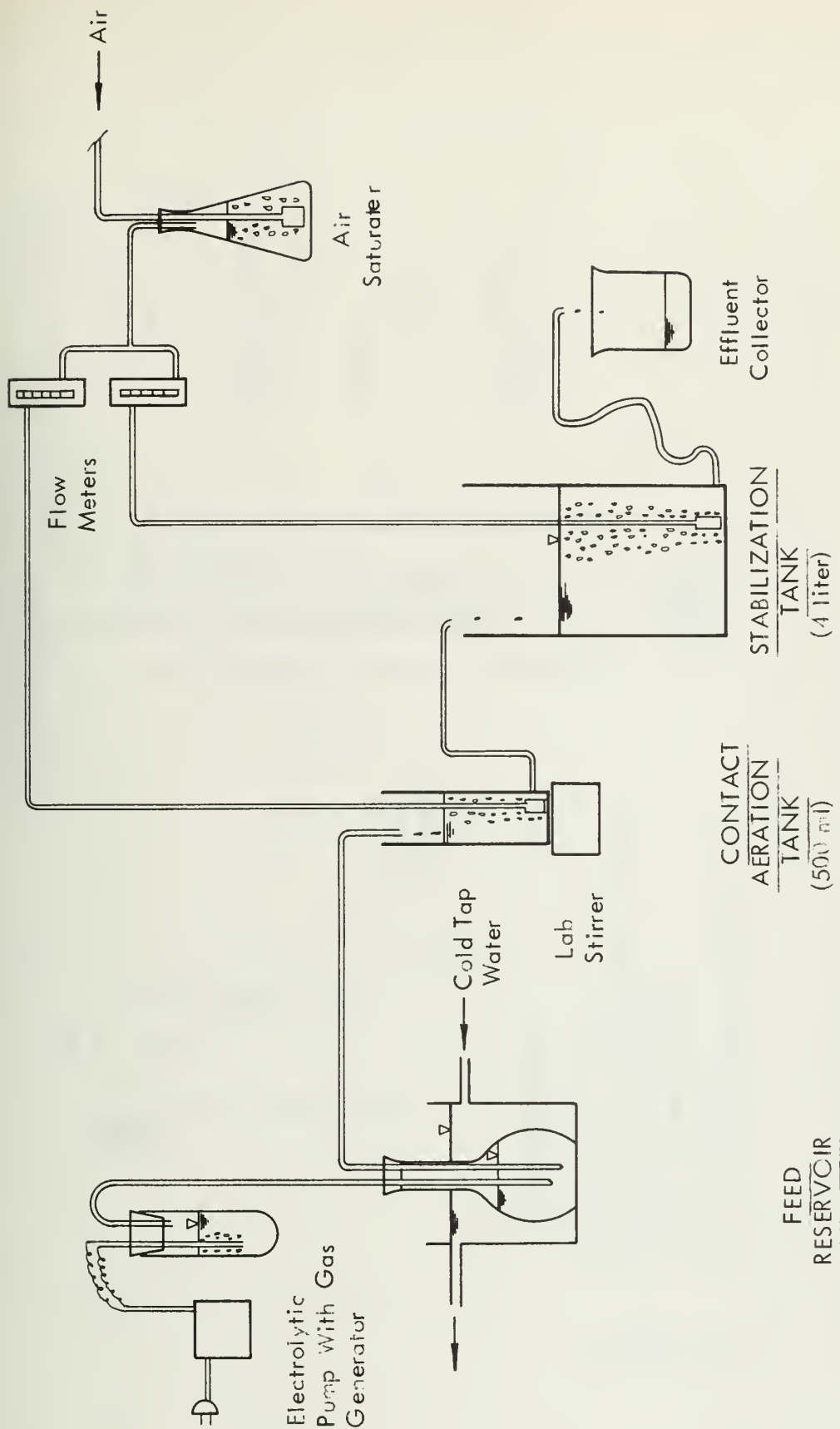
FIGURE 3 CONTINUOUS FLOW LABORATORY ACTIVATED SLUDGE UNIT

To Feed Reservoir

#10 Rubber Stopper

Capillary Tubing (2mm bore)

Mercury

1 + 50 H_2SO_4

Platinum Wire (25 gage x 1")

GAS GENERATOR

To Control Box

R$_1$

M$_1$

To Gas Generator

CR$_1$

c$_1$

CR$_2$

S$_1$

T$_1$

Line Voltage

CONTROL BOX

S$_1$ SPST Switch, 1 amp.
T$_1$ 12.6 v Center Tap Transformer, 3 amp.
CR$_{1,2}$ 500 ma Silicon Rectifier
R$_1$ 300 ohm, 5 watt Rheostat
M$_1$ 0-500 ma Meter
c$_1$ 50 uf, 25v Electrolytic Capacitor
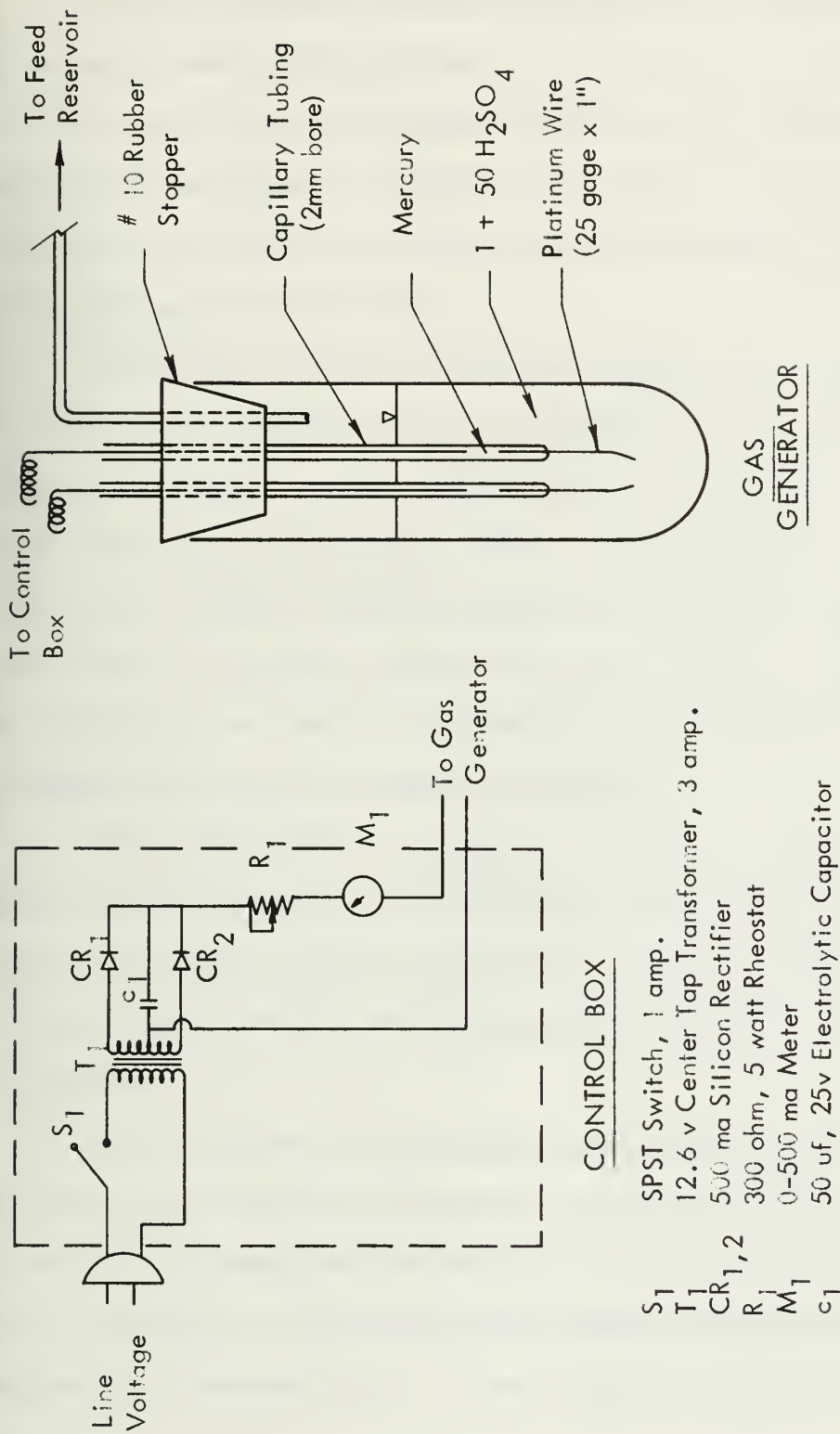
FIGURE 4 ELECTROLYTIC PUMP WITH GAS GENERATOR

aeration tank at the desired rate of 140 ml/hr (3.28 l/day).

The feed reservoir was kept in a water bath through which cold tap water continuously circulated at a temperature of approximately 17° C. The water bath was a necessary adjunct as it prevented excessive growth in the feed solution. Without it, the feed solution would become cloudy within three hours. The feed tube was fashioned from 8-mm capillary tubing with a 2-mm bore.

The aeration vessel, 3 inches in diameter and 8 inches high, was fitted with an overflow tube set to provide a 500-ml aeration volume. Throughout the studies in which the continuous flow unit was used, the aeration volume was never changed. With an average overflow rate of 140 ml/hr the theoretical detention time in the aeration compartment was 3.65 hours. Laboratory compressed air was supplied through a fritted glass diffuser at the rate of 3.0 liter/(min)(liter MLSS) which is more than adequate to maintain an aerobic environment.

The stabilization tank, a glass cylinder 5.75 inches in diameter and 12 inches high, had an overflow which could be set at any desired level between zero and four liters. Air was supplied through two fritted glass diffusers at the rate of 1.0 liter/(min)(liter MLSS) under aeration.

As in the case of the synthetic sewage used for the fill and draw unit, it was desirable to maintain a population in the continuous flow unit close to what would be present in a municipal waste treatment plant. Accordingly, seed organisms were obtained from the Urbana-Champaign Waste Treatment Plant and the synthetic waste shown in Table 6

(except for the sodium acetate) was fed continuously. After a 10-day

period of acclimation, the mixed liquor in the aeration tank was analyzed

for PHB. None could be found. When the unit was restarted using seed

organisms from the fill and draw stock unit, which was definitely known

to contain PHB, the same results were obtained: there was no PHB in the

aeration tank. By adding sodium acetate to the feed substrate, however,

it was possible to observe a slight amount of PHB in the aeration tank

and, therefore, this organic nutrient was made a part of the synthetic

waste for the continuous flow laboratory activated sludge system.

TABLE 6

CONCENTRATION OF SUBSTRATE AND INORGANIC SALTS USED
IN THE FEED RESERVOIR OF THE CONTINUOUS FLOW
ACTIVATED SLUDGE UNIT

	mg/l
Glucose	1,500
Yeast Extract	1,500
Sodium Acetate	1,087
Ammonium Sulfate	250
Magnesium Sulfate (7 waters)	50
Manganous Sulfate (1 water)	2.5
Sodium Chloride	1.0
Calcium Chloride (2 waters)	3.3
Ferric Chloride (6 waters)	0.5
Potassium Phosphate (Dibasic)	1,242
Potassium Phosphate (Monobasic)	221

The addition of sodium acetate also provided a source of fatty acids for

the synthetic waste. The concentration of organic and inorganic nutrients

in the reservoir are shown in Table 6. Trace minerals were supplied by

making up the feed solution with tap water. The pH of the solution was

7.2. Using the COD conversion factors previously mentioned, for glucose and yeast extract plus that for sodium acetate (0.672 gm/gm of sodium acetate) the COD content of the feed was 3795 mg/l and the total nitrogen content, 167 mg/l. The COD/N ratio was, therefore, 22.7.

All organic feed stock solutions were refrigerated after preparation and discarded every two weeks regardless of their condition.

B. Experimental Procedures

1. General Procedure for Fill and Draw Units

Mixed liquor to be used in each experiment was harvested 20 to 23 hours after the unit had received its daily feeding. The mixed liquor was centrifuged and the solids washed with 0.03 molar potassium phosphate buffer, pH 7.2. All washings described in the following pages were carried out using this buffer solution. They were centrifuged a second time and then resuspended in phosphate buffer by homogenizing for 3 minutes with a Sorvall Omni-Mixer Homogenizer set at a power of 80. The purpose of homogenization was to prevent clumping and to insure that a uniform sample could be obtained for analysis. As judged by the rapid response to substrate feeding, the organisms were not adversely affected by homogenization. The sludge was then brought up to the required volume with inorganic nutrients, buffer and tap water and aerated for 10 minutes. At this time samples were removed for zero-hour analysis. The organic substrate was then added and the experiment begun.

In general, samples were collected, centrifuged, washed with buffer or distilled water, centrifuged again and the remaining solids

frozen at -48° C until the end of the experiment when all samples were analyzed. In many cases appropriate dilutions were made just prior to freezing. Variations of this sampling procedure were employed and are discussed below.

2. Food to Microorganism Ratio Studies

On the day of the experiment, 1 liter of mixed liquor was harvested and the sludge solids allowed to settle. After the supernatant was discarded, an equal volume of 0.03 M phosphate buffer was added and the settling and decantation repeated. The remaining sludge was then homogenized prior to its addition to the experimental aeration unit. The sludge solids along with inorganic nitrogen, mineral salts and buffer were diluted to 2 liters with tap water and then aerated for 10 minutes at which time two zero-hour samples were obtained. The carbon sources, glucose and yeast extract, were added in the amounts shown in Table 8 and the experiment begun.

Single samples were collected periodically and processed according to Figure 5. A 40-ml sample of mixed liquor was placed into a stainless steel test tube using a 5-ml Cornwall automatic syringe. The mixed liquor was centrifuged, and the solids washed with buffer and resuspended by homogenization in 40 ml of buffer. Samples were then distributed for carbohydrate, PHB, protein, and solids-COD analyses. At the same time as the 40-ml sample was taken, a 25-ml sample was also obtained using the Cornwall syringe. This sample was centrifuged and

```
                          ┌─────────────────┐
                          │  MIXED LIQUOR   │
                          └─────────────────┘
             40 ml                              25 ml
          centrifuged                        centrifuged

            i.s.*                      soluble        i.s.*
                                                      SOLIDS
1) resuspended with phos.             filter
   buffer using a vortex              through 0.45 u
   mixer                              membrane filter

2) centrifuged
                                      soluble
                                      COD
            i.s.*
       homogenize with 40 ml
       buffer and sampled as
       follows:

            ├── Carbohydrates
            │    5 ml to 100 ml vol. flask and frozen

            ├── PHB
            │    10 ml to heavy wall cent. tube and frozen

            ├── Protein
            │    10 ml diluted to 50 ml; 1.0 ml of dilution
            │    placed in test tube and frozen

            └── Solids-COD
                 5 ml placed directly into COD flask
                 containing COD reagents
```

* i.s. = insoluble fraction

Figure 5   SAMPLING PROTOCOL FOR F/M RATIO STUDIES

the supernatant filtered through a 0.45 micron membrane filter for later COD analysis. By using small quantities of distilled water and a vortex mixer, the solids were transferred to a tared aluminum moisture dish and placed in the oven for drying.

3. COD to Nitrogen Ratio Studies

The procedure for an experiment consisted of harvesting one liter of mixed liquor at the end of a feed cycle and centrifuging at 10,400 x g for 15 minutes. The solids were washed with 0.03 M phosphate buffer, centrifuged again and homogenized for 3 minutes. The homogenized solids, along with inorganic nitrogen, mineral salts and buffer were diluted to 2 liters in a volumetric flask and transferred to the test aeration unit. After aerating for 10 minutes, the zero-hour sample was obtained and the organic substrate then added.

The procedure for sampling was the same as that used in the study on F/M ratio with the exception that the solids were frozen in a polyethylene centrifuge tube until the end of the experiment. They were then transferred to tared aluminum moisture dishes. All solids determinations were, therefore, dried for the same period of time.

4. Substrate Studies

The procedure for these studies was identical to the procedures used in the COD/N Ratio Studies.

5. Studies on the Removal of Substrate During Stabilization

One liter of mixed liquor was obtained near the end of the feeding cycle and centrifuged at 10,400 x g for 15 minutes. The solids

were washed with 0.03 M phosphate buffer, resuspended in phosphate buffer and homogenized for three minutes. The sludge was then placed in an aeration unit to which was added the following: glucose 1000 mg/l, yeast extract 1000 mg/l, $(NH_4)_2SO_4$ 1000 mg/l, mineral salts and buffer according to Table 5, and tap water to dilute the volume to 2 liters. After aerating the mixed liquor for 1.25 hours, it was centrifuged and the solids washed with buffer and resuspended by homogenization for 1 minute. Mineral salts, buffer and nitrogen, in the same concentrations as above, were added to the homogenized solids and the contents diluted to 2 liters with tap water. Aeration was begun and duplicate zero-hour samples obtained immediately.

The sampling procedure was essentially the same as in previous experiments with minor modifications as shown in Figure 6. A 25-ml sample of mixed liquor was removed, centrifuged at 12,100 x g for 10 minutes and the sediment frozen at -48° C for dry weight solids determinations. A second sample, 40 ml, was removed and immediately homogenized for 30 seconds. The sample was then divided into samples for the determination carbohydrate, PHB, protein, and solids-COD. A third sample, 20 ml, was transferred from the aeration tank to a 38 x 200 mm pyrex tube which contained 20 ml of substrate. The final 40-ml volume contained 1000 mg/l COD, equally divided between glucose and yeast extract, as well as inorganic nitrogen, mineral salts and buffer. The mixed liquor suspended solids content varied from 700 to 900 mg/l. The pyrex tube was aerated for 20 minutes

```
                    ┌─────────────────┐
                    │  Mixed Liquor   │
                    └─────────────────┘
```

25 ml
centrifuged

20 ml
added to test tube
containing 20 ml
of substrate and
nutrients. Solution
aerated for 20 min.

SOLIDS

40 ml
Homogenized and
sampled as
follows

centrifuged

— Carbohydrates
5 ml into 100 ml volumetric flask
and frozen

Supernatant filtered
through 0.45 u filter

— PHB
10 ml into a heavy wall cent. tube
and frozen

— Protein
10 ml diluted to 50 ml; 1.0 ml of
dilution placed in test tube and
frozen

SOLUBLE
COD
REMOVED IN
20 MIN. CONTACT
PERIOD

— Solids - COD
5 ml placed directly into COD flask
containing COD reagents

Figure 6   SAMPLING PROTOCOL FOR THE STUDY RELATING TO THE
INFLUENCE OF STORAGE PRODUCTS ON COD REMOVAL

using a 9-inch disposable pipette. At the end of the 20-minute
contact period, the contents were poured into a pre-frozen stainless
steel centrifuge tube (Sorvall #517F) and immediately centrifuged at
12,100 x g for 8 minutes at a temperature of 0° C. The supernatant
was filtered through a 0.45 micron membrane filter and stored in the
refrigerator until analyzed. All samples were run in duplicate.

C. Analytical Techniques

   1. Mixed Liquor Suspended Solids

        Although the membrane filter technique for mixed liquor
suspended solids (MLSS) determination is quite simple and easy to
use, it was highly unsatisfactory for use in these studies. Samples
of only 5 ml required up to 30 minutes to filter through a 0.45
micron membrane filter. This was particularly true for mixed liquor
samples obtained from the aeration tank of the continuous flow unit.
During this time the organisms were still in contact with substrate
and would continue to metabolize and grow. The error thus introduced
could be substantial especially during the early period of substrate
contact. Consequently, it was decided to employ centrifugation for
solids analysis.

        All solids determinations were carried out on the pellet
obtained from a 25-ml sample of mixed liquor that had been centrifuged
and the sludge washed once with distilled water. The centrifuging
was performed using a Sorvall RC-2 refrigerated unit operated at 0° C.

The pellet was usually frozen and then transferred into a tared aluminum planchet, dried for 24 hours at 100° C, cooled fifteen minutes and reweighed.

McWhorter and Heukelekian (10) found greater than 99 per cent removal of bacterial cells when they centrifuged at 32,000 x g; however, in the present work this speed produced a pellet that was extremely difficult to remove from the centrifuge tube. A comparison between the centrifuge technique and membrane filtration indicated that centrifugation at 7710 x g gave a solids concentration within 10 mg/l of that obtained by the membrane filter method when the MLSS concentration was approximately 2200 mg/l.

2. Chemical Oxygen Demand

The Chemical Oxygen Demand (COD) of soluble as well as solid material was determined in accordance with a modified version of the Standard Methods procedure (82). The modified procedure, as prepared by the COD subcommittee of the Standard Methods Committee, Water Pollution Control Federation (Jan. 1964), involves the following quantities of reagents and sample:

    20 ml of sample or aliquot diluted to 20 ml

    10 ml of 0.25N potassium dichromate

    30 ml of concentrated sulfuric acid with 22 gms of silver
            sulfate added per nine-pound bottle of acid

    0.10N ferrous ammonium sulfate

    0.4 gm mercuric sulfate per sample

The mercuric sulfate addition followed the recommendation of Dobbs and Williams (83) for the elimination of the chloride interference.

Samples were refluxed for two hours and the dichromate backtitrated with ferrous ammonium sulfate using ferroin indicator to determine the endpoint.

Carbon Dioxide evolution was determined by the direct method as described in Manometric Techniques (84). This method involves the use of duplicate Warburg flasks, one of which contains the CO_2 absorbent, potassium hydroxide, and the other in which no absorbent is used. The difference in pressure between the two flasks along with appropriate CO_2 and O_2 flask constants is a measure of the CO_2 evolved.

Carbohydrates

Cell carbohydrates were determined according to the Anthrone Method of Scott and Melvin (85). The procedure was followed exactly except for the water bath temperature which was 100° C rather than 90° C. After washing and homogenizing the solids in 0.03 molar buffer, appropriate dilutions were made immediately and the sample stored at -48° C for later analysis. The thawed sample was mixed with cold anthrone reagent using a 10-ml Cornwall Automatic Syringe and Vortex mixer and placed in a boiling water bath for 15 minutes, cooled, and the optical density read on a Beckman DU Spectrophotometer against

a distilled water-anthrone blank. Gaudy (51,86) has adequately reviewed the history of the test.

Glucose, preserved with 0.6 ml of commercial "Roccal"* per liter of glucose solution, served as the standard. The presence of "Roccal" allowed a stock solution of glucose to be stored at room temperature indefinitely. The standard curve of color development as well as the maximum absorbance for anthrone is shown in Figure 7. Throughout these studies a wavelength of 625 mu was used.

5. Protein

All protein measurements were made according to the modified Folin method outlined by Orme-Johnson and Woods (87), which was based on the work of Lowry et al. (88). Of the two commonly used tests for protein, the Biuret and the Folin, the latter was chosen because 1) there is no apparent interference by the ammonium ion or other substances found in sewage sludges, 2) it is 100 times more sensitive than the Biuret test thus affording the use of smaller size samples, and 3) it is more rapid and easier to perform. The test involves the reaction of Folin Phenol Reagent with tryosine and tryptophan amino acid units of a protein molecule and results in the formation of a blue colored complex.

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*Roccal (10 per cent solution) - A product of Winthrop Laboratories, New York. Active ingredients: alkyldimethylbenzylammonium chloride (C_{12}, C_{14}, C_{16} and other related alkyl groups from C_8 to C_{18}) $\pm$ 10 per cent. Inert ingredient: water, 90 per cent.
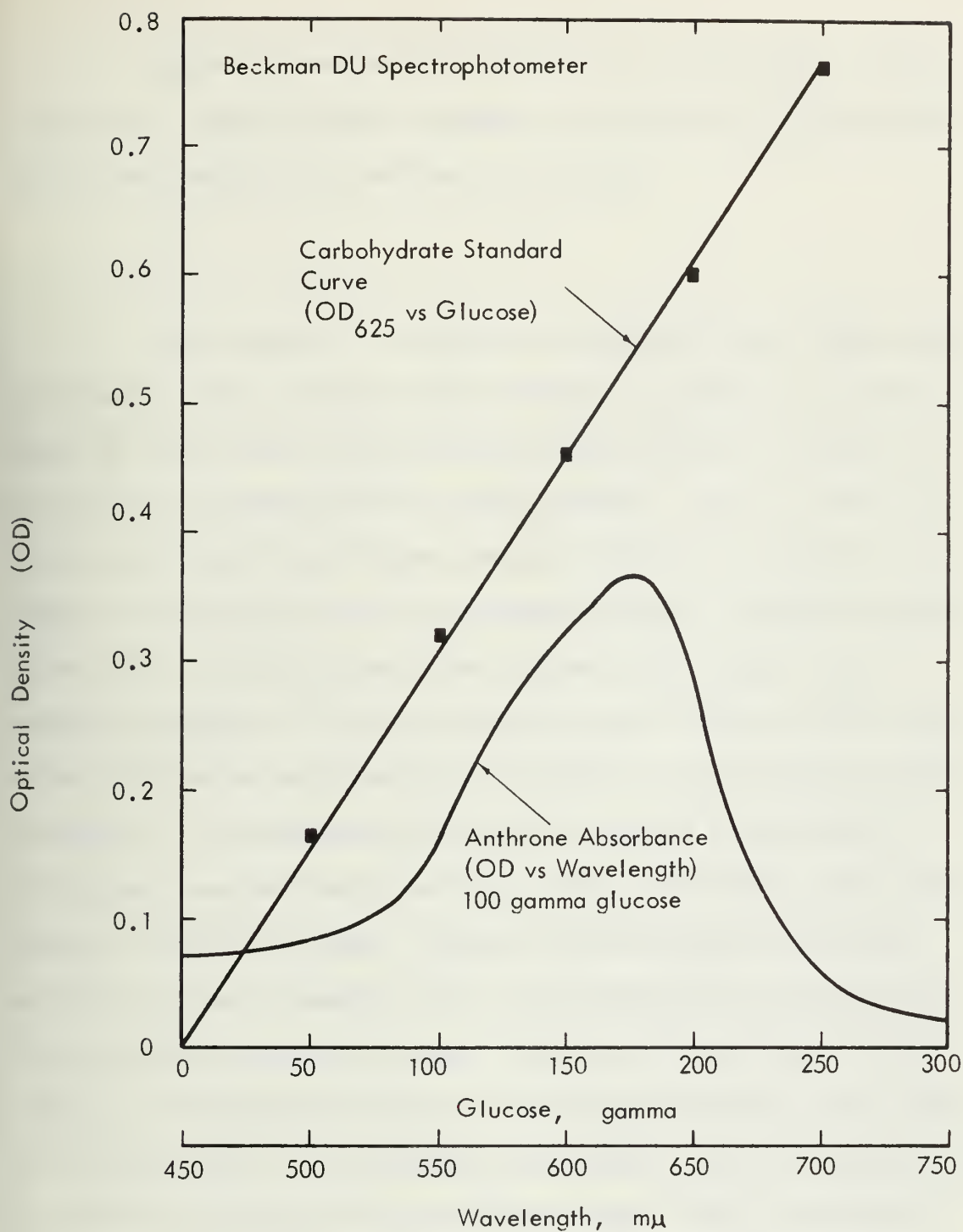
FIGURE 7   MAXIMUM ABSORBANCE AND STANDARD CURVE FOR
ANTHRONE CARBOHYDRATE TEST

Maximum absorbance was found to be at a wavelength of 750 mu (Figure 8). Sample dilutions were made so the optical densities would fall on the straight line portions of the curve.

Early analysis of the poly-beta-hydroxybutyrate (PHB) content of bacterial cells by Lemoigne (58) involved a gravimetric procedure in which the polymer was extracted with boiling chloroform subsequent to the removal of contaminants with other solvents. This was a long and tedious assay requiring milligram amounts of PHB to be present in the sample. In addition to the time consuming aspect, it was not a precise method (89), In 1958 Williamson and Wilkinson (61) devised a more sensitive procedure in which they dissolved the cellular material with sodium hypochlorite and measured the turbidity of the resultant lipid inclusions. By standardizing the turbidity against the quantity of PHB obtained gravimetrically they were able to considerably reduce the amount of time for the assay. However this method was used on pure cultures and they recommended such a standardization be repeated each time a different organism was used. Inasmuch as the activated sludge system is a heterogeneous culture it was assumed that this method would not be satisfactory. An experiment was designed to test the applicability of this latter procedure using activated sludge. PHB, as measured by the turbidity of the lipid inclusions, was compared to PHB obtained by a spectrophotometric method (described in the next paragraph). The former method did not show any correlation with the latter and was

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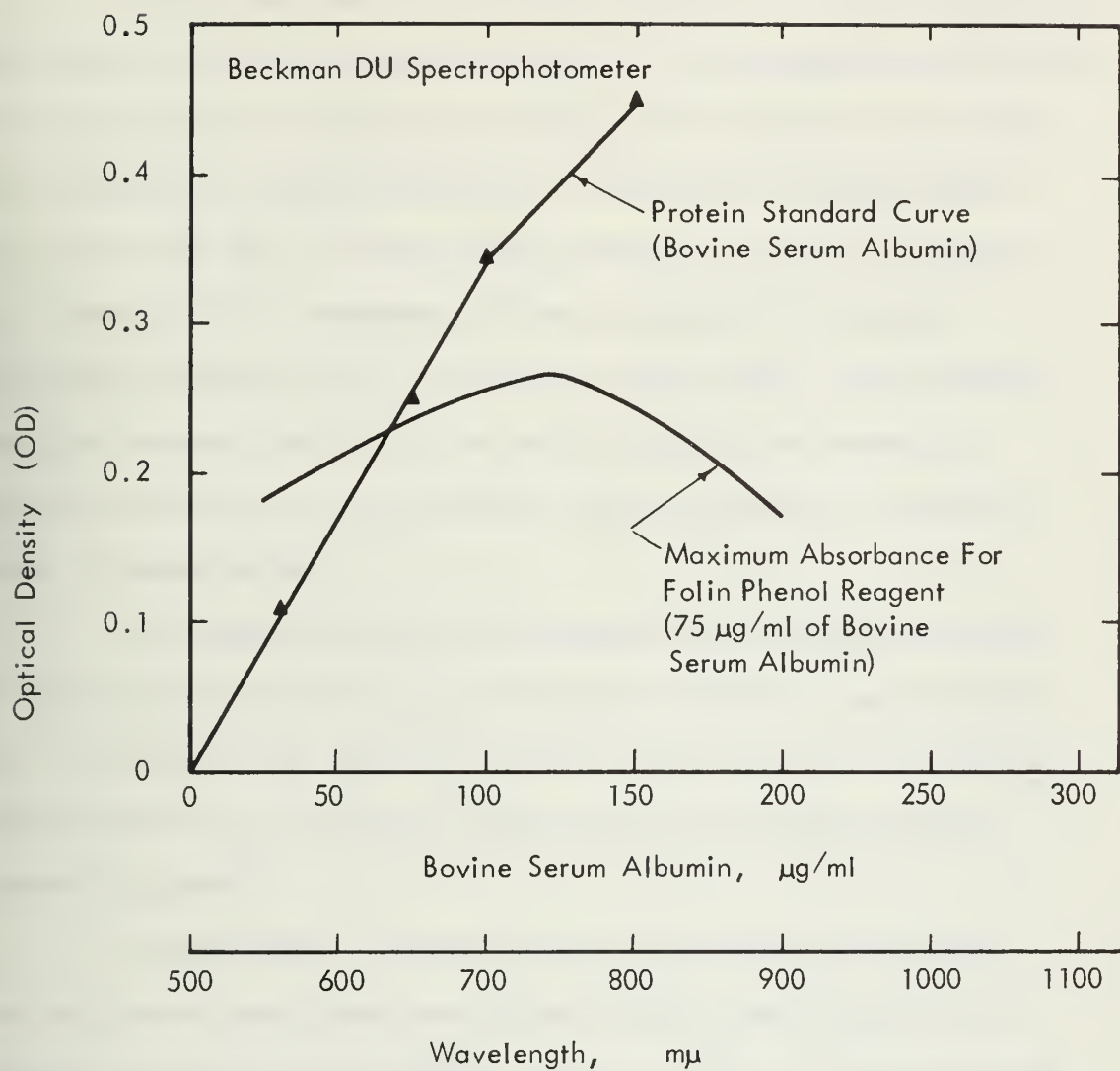
FIGURE 8   MAXIMUM ABSORBANCE AND STANDARD CURVE FOR THE
FOLIN PROTEIN TEST

consequently not considered for use in this work.

In 1961 Law and Slepeckey (60) introduced a spectrophotometric method that involved isolating the polymer by chloroform extraction and then converting it to crotonic acid which can be measured in the ultra-violet region of a spectrophotometer. The principle of this method lies in the fact that the ultraviolet absorption maxima of an alpha or beta unsaturated acid undergoes a strong bathochromic shift when concentrated sulfuric acid is used as the solvent (90). The resultant absorption maximum lies within the useful range of the Beckman DU Spectrophotometer and is sufficiently intense to provide a sensitive method of determination.

The assay technique used throughout this work incorporated the procedures outlined by Law and Slepeckey (89) with some modifica-tions. Since it is the first time such a test has been used with the organisms found in an activated sludge system, the entire assay is presented in detail.

A 10-ml sample of mixed liquor was pipetted into a 12-ml heavy wall pyrex centrifuge tube (Sorvall #102A) containing 2 ml of commercial Clorox* and immediately frozen at -48° C. At the end of an experiment, all samples were thawed in warm water and centrifuged at 7710 x g for eight minutes. The pellet was resuspended in 10 ml of Clorox and placed in a 37° C water bath for 1 hour. The tube was then

*Clorox: 5.25% Sodium Hypochlorite solution; a commercial product of the Clorox Company, Oakland, California

centrifuged and the solids washed with 10 ml of distilled water and
again centrifuged. Finally the solids were washed with 10 ml of acetone
and centrifuged for 15 minutes at 17,300 x g. The supernatant was
discarded and the solids were allowed to air dry for several hours
before continuing. This last step was sometimes expedited by directing
a stream of air into the centrifuge tube.

The PHB was then solubilized by placing 10 ml of chloroform
into the centrifuge tube, capping with a rubber cap (Sorvall #321) and
incubating the mixture in a 58° C water bath for fifteen minutes. The
chloroform extract was then passed through a sintered glass filter
(medium porosity) and the filtrate used for the photometric assay.
There was some question as to whether the rubber cap would interfere
with the analysis; however, it was unfounded.

A dilution of the filtrate was made (usually 2 ml of filtrate
to 98 ml of chloroform) and 5 ml of the dilution placed in a clean test
tube and evaporated in a 75° C water bath. When all the chloroform
had disappeared 10 ml of concentrated sulfuric acid were added, the
contents capped and all tubes placed in a boiling water bath for 10
minutes. The solution was cooled and after thorough mixing, a sample
was placed in a silica cuvette. The absorbance was measured at 235 mu
against a sulfuric acid blank which had been in the boiling water bath
for 10 minutes.

The amount of polymer was determined using a molar extinction
coefficient of 1.44×10^4 which was calculated from the curve obtained

using purified PHB isolated from twenty liters of activated sludge mixed
liquor (Figure 9). A molecular weight of 86 was used for the depoly-
merized PHB. This extinction coefficient was in close agreement with
that published by Law and Slepeckey, 1.56×10^4 (90).

On some occasions purified PHB was obtained in a larger
quantity. For the isolation of large quantities of the polymer
essentially the same procedure was used except for certain modifications.
All washings were performed by refluxing the solvent and solids for two
hours. The water wash was followed by a washing with 95 per cent
alcohol and then the regular acetone wash. The polymer was precipitated
from the chloroform solution by the addition of eight volumes of acetone
and then placed at -20° C overnight. The precipitate was centrifuged
and redissolved twice in chloroform before it was dried. This prepara-
tion constituted the purified polymer which was used for infrared
analysis, carbon-hydrogen-oxygen analysis, melting point determinations
and for the preparation of the standard curve.

7. Sludge Volume Index

Because of the lack of a sufficient volume of sludge to
make a Mohlman Sludge Volume Index determination, it was necessary to
alter the test procedure. Instead of the one-liter volume normally
used to measure sludge volume, 100 ml were substituted. The volume of
sludge which settled in a 100-ml graduated cylinder in thirty minutes
was multiplied by 10 and divided by the sludge concentration in grams.
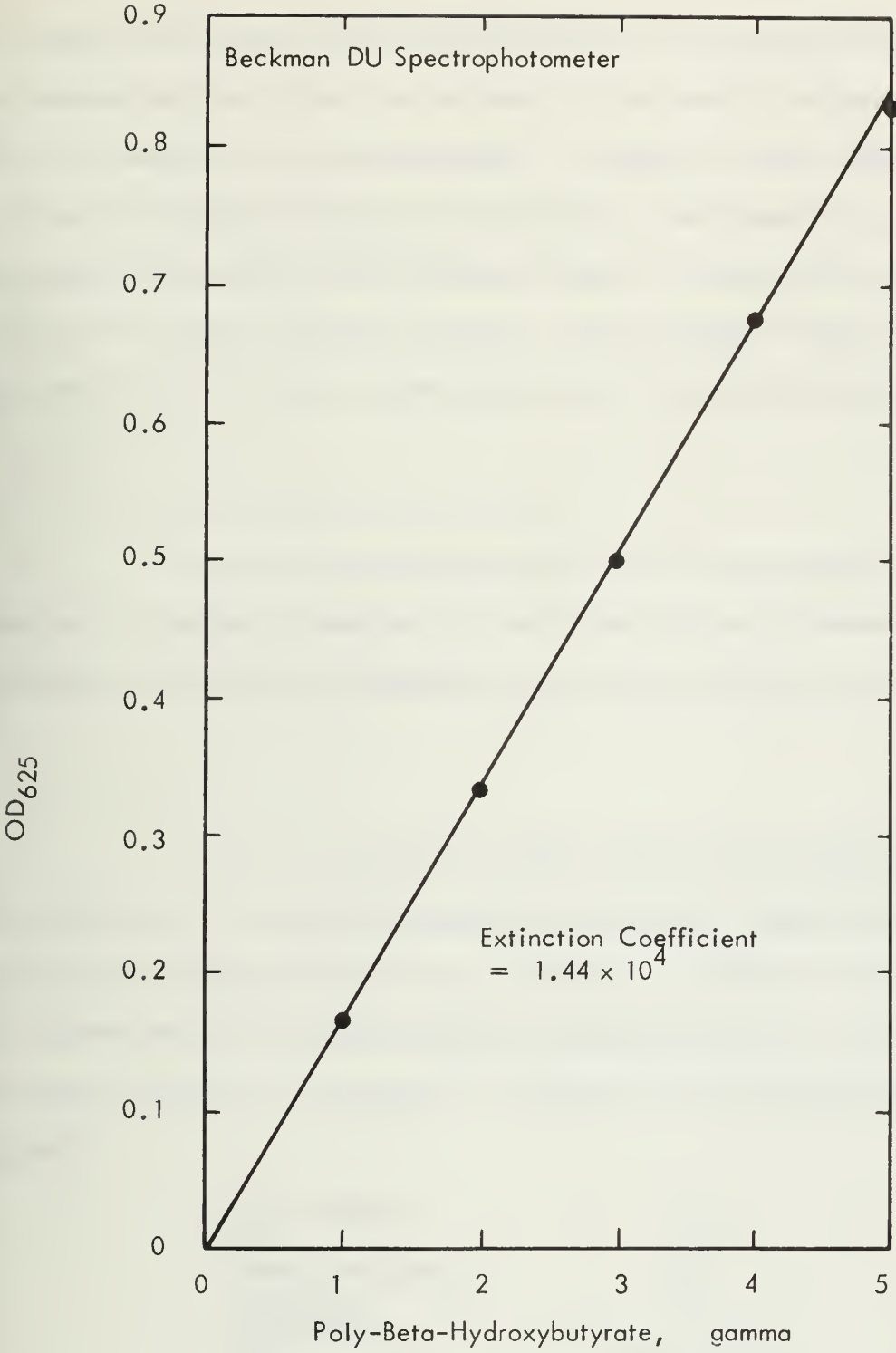
Beckman DU Spectrophotometer

Extinction Coefficient
$= 1.44 \times 10^4$

OD$_{625}$

Poly–Beta–Hydroxybutyrate, gamma

FIGURE 9   STANDARD CURVE FOR POLY–BETA–HYDROXYBUTYRATE TEST

This scaled down sludge volume index provides the same connotation as the standard Mohlman SVI and may be used for comparative purposes within the scope of this investigation. It cannot be used, however, for absolute comparison against SVI reported in the literature for municipal or industrial waste treatment plants. Because of the wall effect in the 100-ml graduated cylinder, this "modified" SVI determination would tend to reflect a lower value than the standard Mohlman Index.

8. Carbon-Hydrogen-Oxygen Analysis

The carbon-hydrogen-oxygen analysis was performed according to standard procedures by the Microanalytical Laboratory, Department of Chemistry and Chemical Engineering, University of Illinois.

9. Infrared Analysis

Infrared analysis of sludge samples and purified PHB were performed in the Infrared Analytical Laboratory, Department of Chemistry and Chemical Engineering, University of Illinois, using a Perkin-Elmer Model 521 Grating Infrared Spectrophotometer. The following information is offered as a matter of record for the samples analyzed:

Slit program	10
Gain	4.5
Attenuator Speed	11
Scan time	16
Suppression	4
Scale	standard
Source current	0.4 amp
Phase	potassium bromide discs
Sample weight	approx. 1 mg

VI. RESULTS AND DISCUSSION

A. Occurrence of Storage Products in Activated Sludge

Prior to studying the significance of substrate storage in an activated sludge system, it was considered necessary to determine if the two major bacterial carbon and energy storage compounds, poly-beta-hydroxybutyrate and glycogen, were actually present in activated sludge obtained from a wastewater treatment plant. Unless their presence was verified, the purpose of this research would lose a great deal of its practical significance.

1. Poly-beta-hydroxybutyrate

a. PHB in Wastewater Treatment Plants and Laboratory Activated Sludge

At the beginning of experimental work, the two literature references (48,50) describing the presence of PHB in Z. ramigera had not been published and it was highly questionable whether this material was actually present.

Initially it was established that three criteria had to be met for the lipid reserve polymer to be considered as being absolutely identified as a component of the sludge from a municipal wastewater treatment plant:

1. the treatment plant sludge must show an infrared absorption band between 5.7 and 5.8 microns. This is the band that has been repeatedly shown to be characteristic of PHB.

2. when treatment plant activated sludge is used as a seed for a laboratory unit, PHB must be synthesized in significant amounts and its presence demonstrated by isolating and characterizing the material.

3. PHB must be isolated from treatment plant activated sludge.

In the isolation procedure for PHB, the first step is to dissolve a major portion of the cellular material with commercial Clorox. Since the PHB is insoluble in Clorox, the step would serve to concentrate the PHB and remove any material that might mask the absorption peak of PHB. To find out if this were true, a sample of activated sludge was obtained from the local Urbana-Champaign (U-C) Sanitary District Wastewater Treatment Plant for infrared analyses. The plant is operated on the contact-stabilization activated sludge principle and the sample was obtained from the middle of the contact unit.

An aliquot was centrifuged, washed with distilled water and resuspended in an equal volume of Clorox. The solution was incubated for one hour at 37° C, centrifuged, washed twice with distilled water and then dried. The dried material was ground in a mortar and approximately 1 mg weighed out for I.R. analysis.

A second aliquot was washed twice with distilled water, dried, ground, and approximately 1 mg weighed out for the I.R. analysis.

Figure 10 shows the influence of Clorox treatment on plant activated sludge. By treating sludge with Clorox a more intense absorption peak was obtained at 5.8 microns. It was, therefore, decided
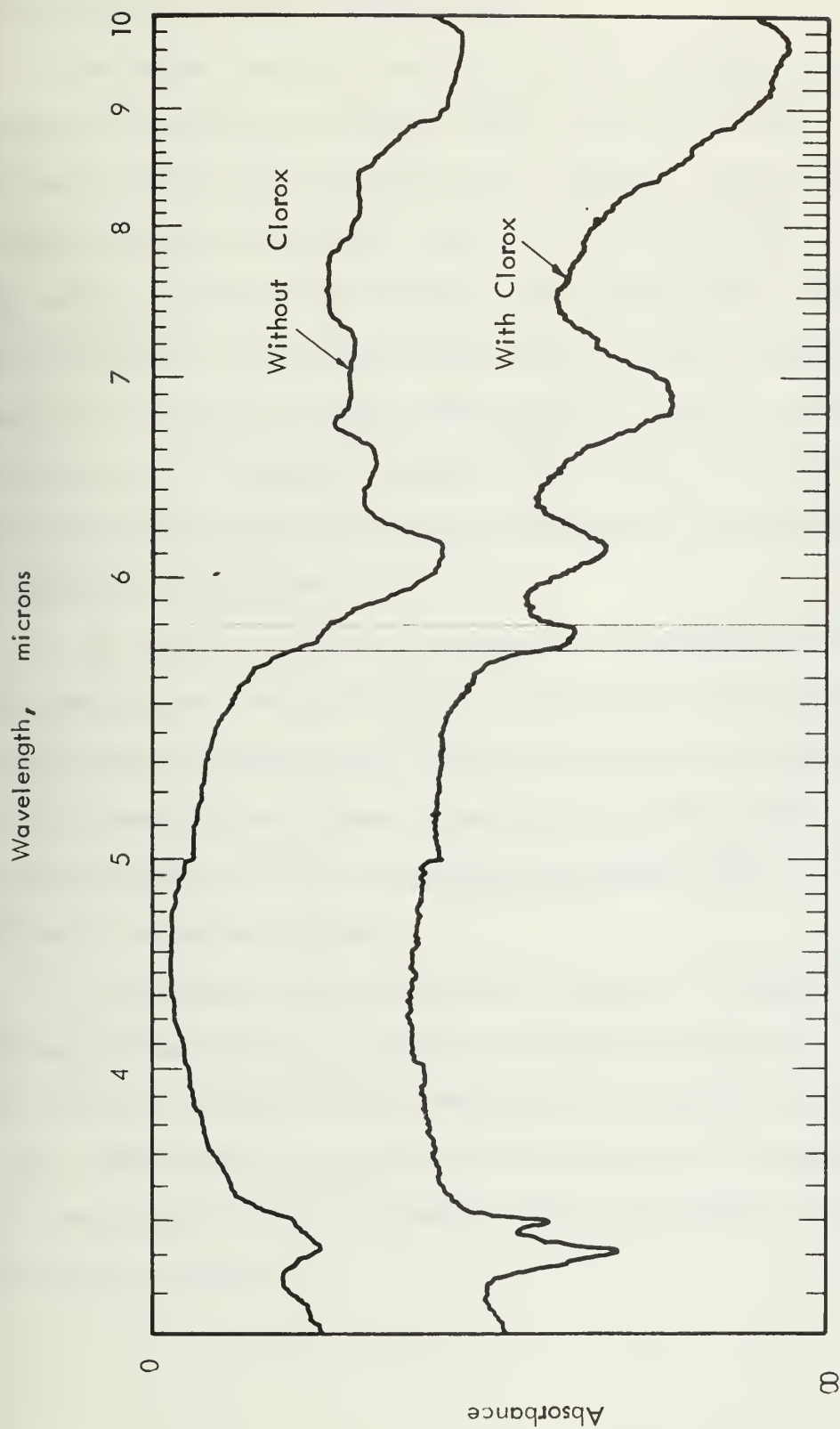
FIGURE 10 INFLUENCE OF CLOROX TREATMENT ON THE INFRARED ABSORPTION SPECTRUM OF ACTIVATED SLUDGE OBTAINED FROM THE CONTACT AERATION UNIT OF THE URBANA-CHAMPAIGN MUNICIPAL WASTEWATER TREATMENT PLANT

to process all plant activated sludge samples by first treating them with Clorox in the manner described above.

The second condition was investigated by seeding a 2-liter laboratory fill and draw activated sludge unit with the same sludge from the U-C plant that was used for I.R. analysis. The unit was fed a mixture of glucose (2000 mg/l) and yeast extract (500 mg/l) using a daily wasting schedule of 50 per cent of the mixed liquor. After five days of acclimation, two samples were removed for I.R. analysis. The samples were obtained 1.5 hours after feeding in order to provide sufficient time for maximum synthesis of the polymer. One sample was treated with Clorox while the other was washed with distilled water in the manner described above.

The results of the I.R. analyses on the laboratory activated sludge samples are shown in Figure 11. The Clorox treated sample showed a more intense absorption peak at 5.8 microns than the untreated sample. Since the absorption was strong using just the washed cells, it was therefore decided not to use Clorox treated samples for future I.R. analyses of laboratory sludges.

A comparison of the non-Cloroxed samples of treatment plant activated sludge solids and laboratory activated sludge solids showed that only the laboratory solids demonstrated an absorption peak at 5.8 microns. The absence of a peak in the spectrum of the treatment plant solids was probably due to the masking effect of material that could be dissolved with Clorox.
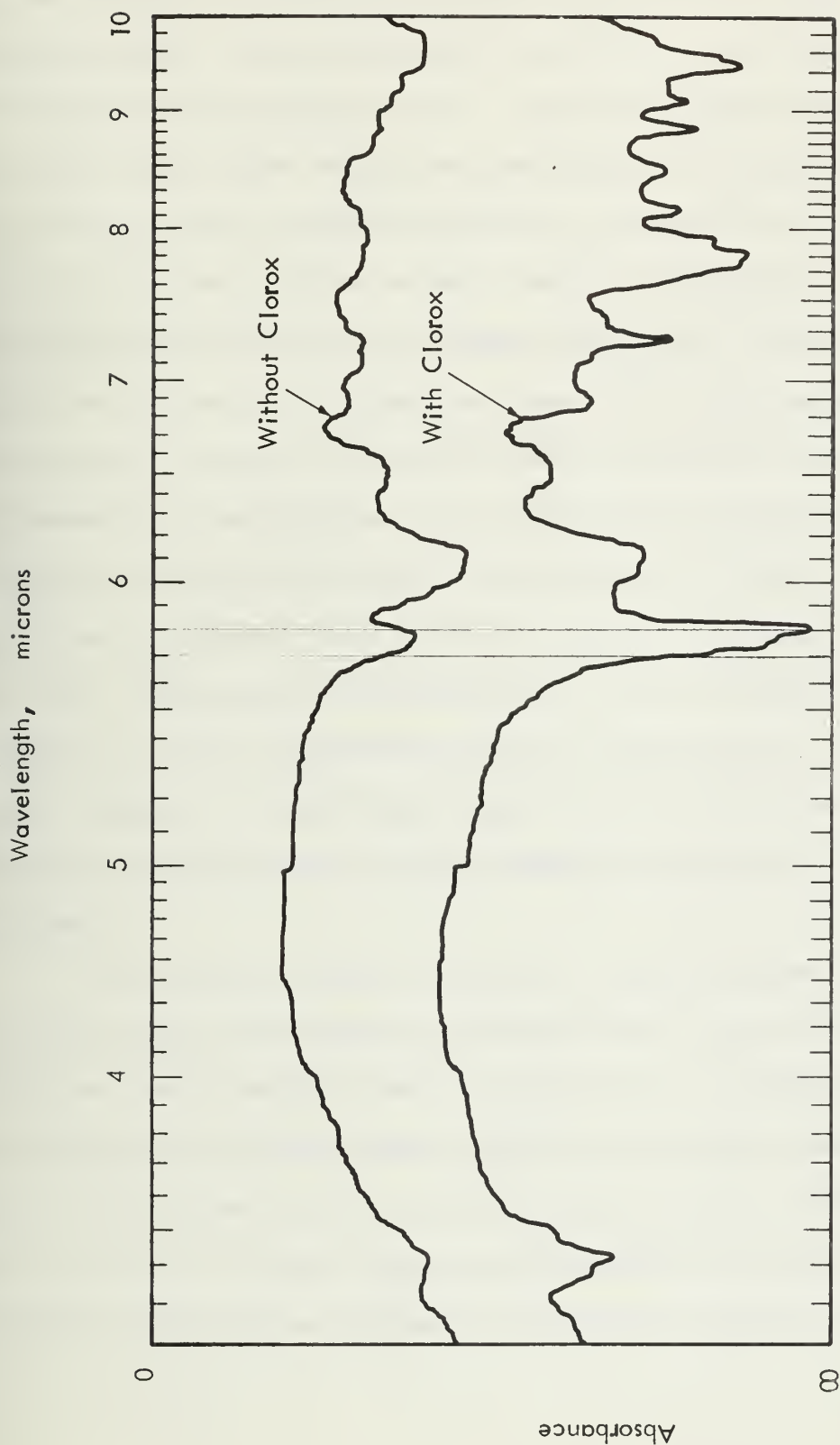
FIGURE 11 INFLUENCE OF CLOROX TREATMENT ON THE INFRARED ABSORPTION SPECTRUM OF LABORATORY ACTIVATED SLUDGE

Further evidence of the existence of PHB in the laboratory unit was obtained by actually measuring the PHB content of the laboratory sludge. After thirty days of acclimation, an experiment was conducted on the laboratory unit. One liter of mixed liquor was harvested 23 hours after feeding and centrifuged. The solids were washed with 0.03 molar phosphate buffer and resuspended prior to addition to the test aeration vessel. The normal daily feed of glucose and yeast extract as well as the minerals and buffer, as shown in Table 5, were added and the experiment begun. The following analyses were performed: soluble COD, soluble glucose, MLSS, PHB of the sludge, and intracellular carbohydrates. The intracellular carbohydrates, an indication of the glycogen content, were measured by treating 20 ml of mixed liquor solids with 20 ml of cold 10 per cent trichloroacetic acid (TCA). The mixture was incubated at 4° C for 30 minutes, centrifuged, and the supernatant assayed for carbohydrates. Soluble glucose remaining in solution was measured by the specific enzyme preparation, Glucostat (Worthington Biochemical Corporation, Freehold, New Jersey).

The results are shown in Figure 12. The PHB content varied from 5.0 per cent of the solids dry weight at time zero to a maximum of 18.5 per cent after three hours. After 14 hours the PHB content had returned to its original value. Glucose was entirely removed from solution within three hours at which time there remained a residual COD in solution. The yeast extract was also removed as judged by the difference between COD and glucose at the beginning and after three hours.
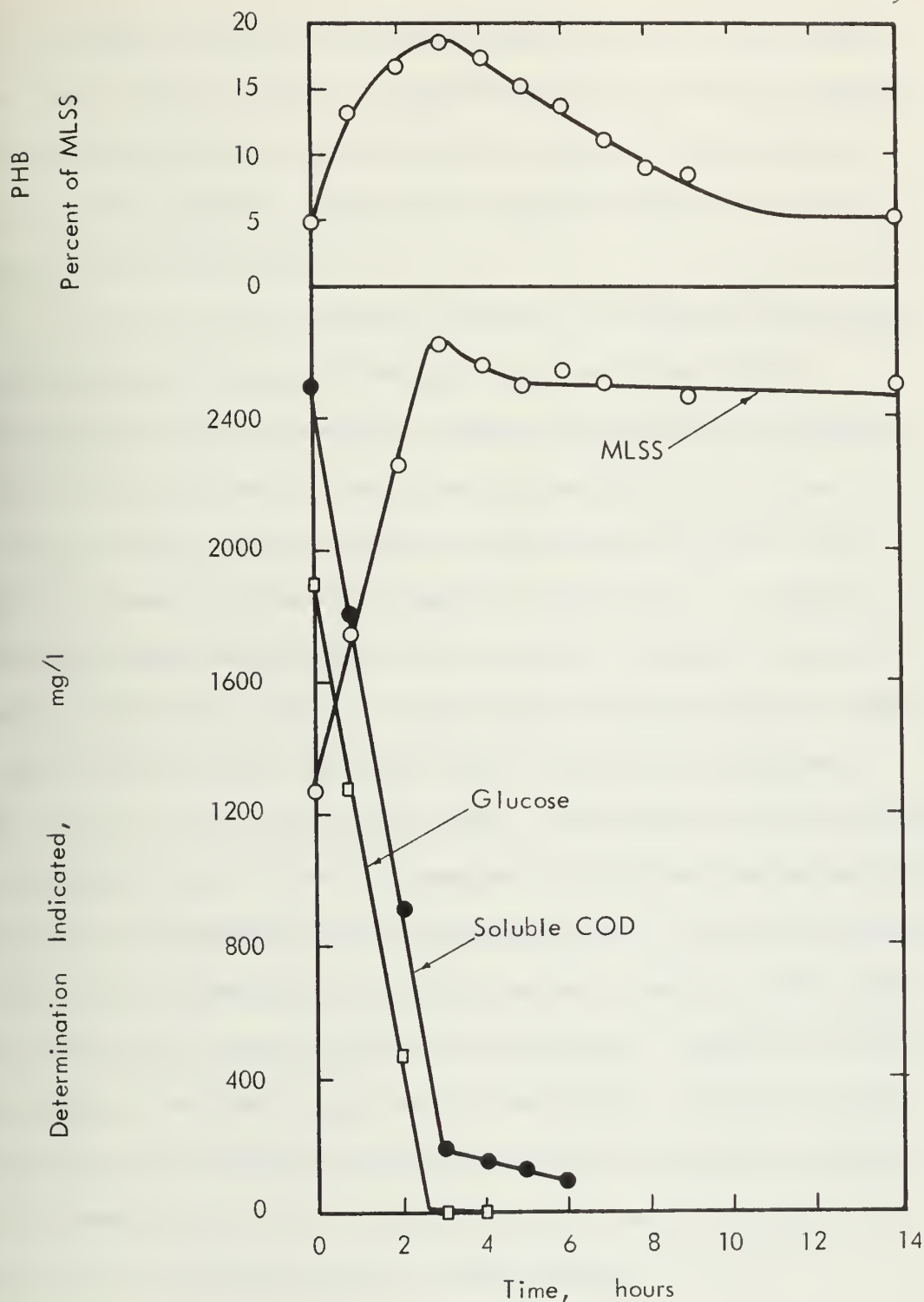
FIGURE 12   VARIATION IN PHB STORAGE DURING METABOLISM OF
GLUCOSE-YEAST EXTRACT BY ACTIVATED SLUDGE FROM THE
STOCK UNIT

If all the yeast extract had not been removed after the first three hours, there would have been at least 600 mg/l COD remaining at that time. The intracellular carbohydrates, a measure of the glycogen content of the organisms, varied only slightly from 0.70 per cent to 0.85 per cent of the solids.

From the results thus far presented, it appeared that PHB was actually a component part of treatment plant activated sludge. Therefore, the third criterion for absolute identification of PHB was studied. Twenty liters of sludge from the contact aeration tank of the U-C wastewater treatment plant were processed according to the isolation procedure outlined in Chapter V, Section C, 6. When the chloroform extract was placed in cold acetone, a negligible amount of pinpoint precipitate formed. A second sample, thirty liters in volume, was again processed with the same results. There was no apparent reason for the lack of precipitable PHB. Its presence had been indicated by I.R. analysis and it could be measured in laboratory activated sludge seeded with the treatment plant activated sludge. If the concentration of PHB in the treatment plant mixed liquor was 1 per cent of the MLSS, 600 mg should have been available after isolation. Therefore, the PHB must have been less than 1 per cent of the sludge. As will be shown subsequently, the location of the sample within the aeration tanks of the activated sludge unit is important and this could have been the reason for the negligible amount of PHB obtained.

Despite the inability to isolate PHB from treatment plant activated sludge, it was felt that fulfilling two of the three criteria constituted enough evidence for the existence of PHB to justify continuing the study by obtaining I.R. analyses on activated sludge solids from other waste treatment plants. Two conventionally operated activated sludge plants, at Mattoon and Tuscola, Illinois, were sampled and the activated sludge solids subjected to Clorox treatment. The I.R. spectrums are shown in Figure 13 along with the U-C treatment plant sludge for comparison. All sludges were obtained from approximately the middle of the aeration tank. The Tuscola plant sludge showed only a very slight peak at 5.8 microns; it would take further work to establish definitely the existence of PHB in the sludge from this plant. The results of the U-C treatment plant I.R. spectrum have already been discussed and it was concluded that PHB was present in the sludge. The sample from the Mattoon plant was quite interesting. The absorption intensity was stronger than that for protein which is approximately 6.1 microns according to Levine et al. (91). This was not true for any of the other activated sludge samples. Even in the nontreated laboratory samples, the PHB peak was never more intense than the protein peak. It was interesting to note that the Mattoon sludge was the only one of the three sludges that had extremely poor flocculation and settling properties. Some of the solids settled but the supernatant was highly turbid. This fact was not investigated further.
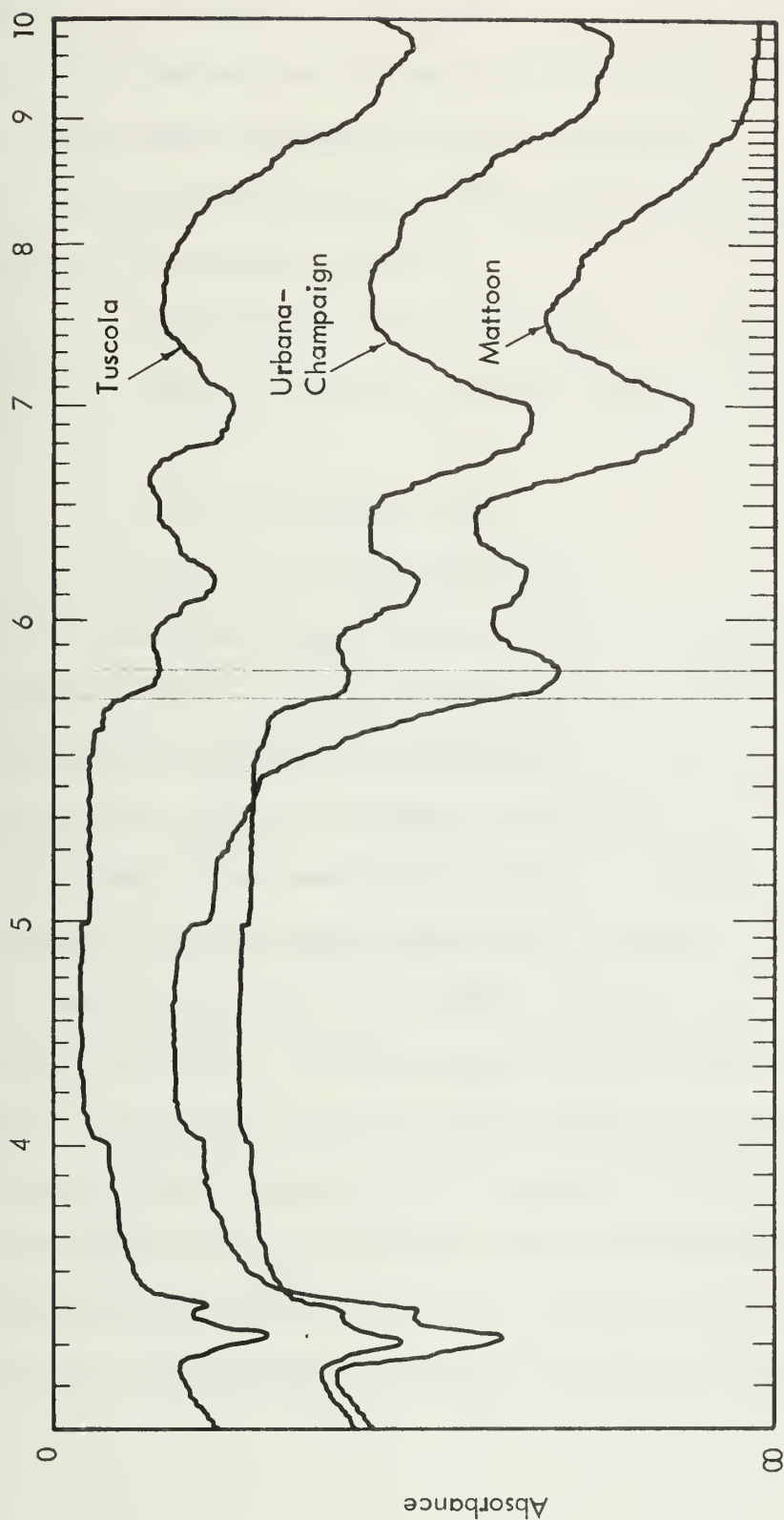
FIGURE 13   INFRARED ABSORPTION SPECTRUM OF ACTIVATED SLUDGE OBTAINED FROM THE
AERATION TANK OF THREE ACTIVATED SLUDGE WASTEWATER TREATMENT PLANTS
IN THE STATE OF ILLINOIS

If PHB is synthesized and subsequently degraded by activated sludge organisms, the intensity of absorption at 5.8 microns should change with samples obtained from different locations throughout the process, i.e., the contact aeration and stabilization tanks. Accordingly, sludge samples were obtained from the U-C wastewater treatment plant at the following locations:

1. beginning of the contact tank
2. twenty per cent of the way through the contact tank
3. end of the contact tank
4. end of the stabilization tank

The I.R. spectrum for each sample is shown in Figure 14. At a point twenty per cent of the way through the contact tank, a significant absorption intensity was demonstrated. At the beginning and end of the contact tank, the absorption intensities were virtually the same; in both samples the absorption decreased in intensity between 5.8 and 5.9 microns. In the sample obtained from the end of the stabilization tank the spectrum did not show a decrease in absorption between 5.8 and 5.9 microns. It would appear from these data that a major portion of the synthesis and subsequent degradation of PHB occurred during the contact period in this wastewater treatment plant. The data also indicate the type of pattern that could be expected for a sludge that has undergone stabilization, i.e., a pattern that is not totally devoid of a distinguishable "dip" at 5.8 microns. This could
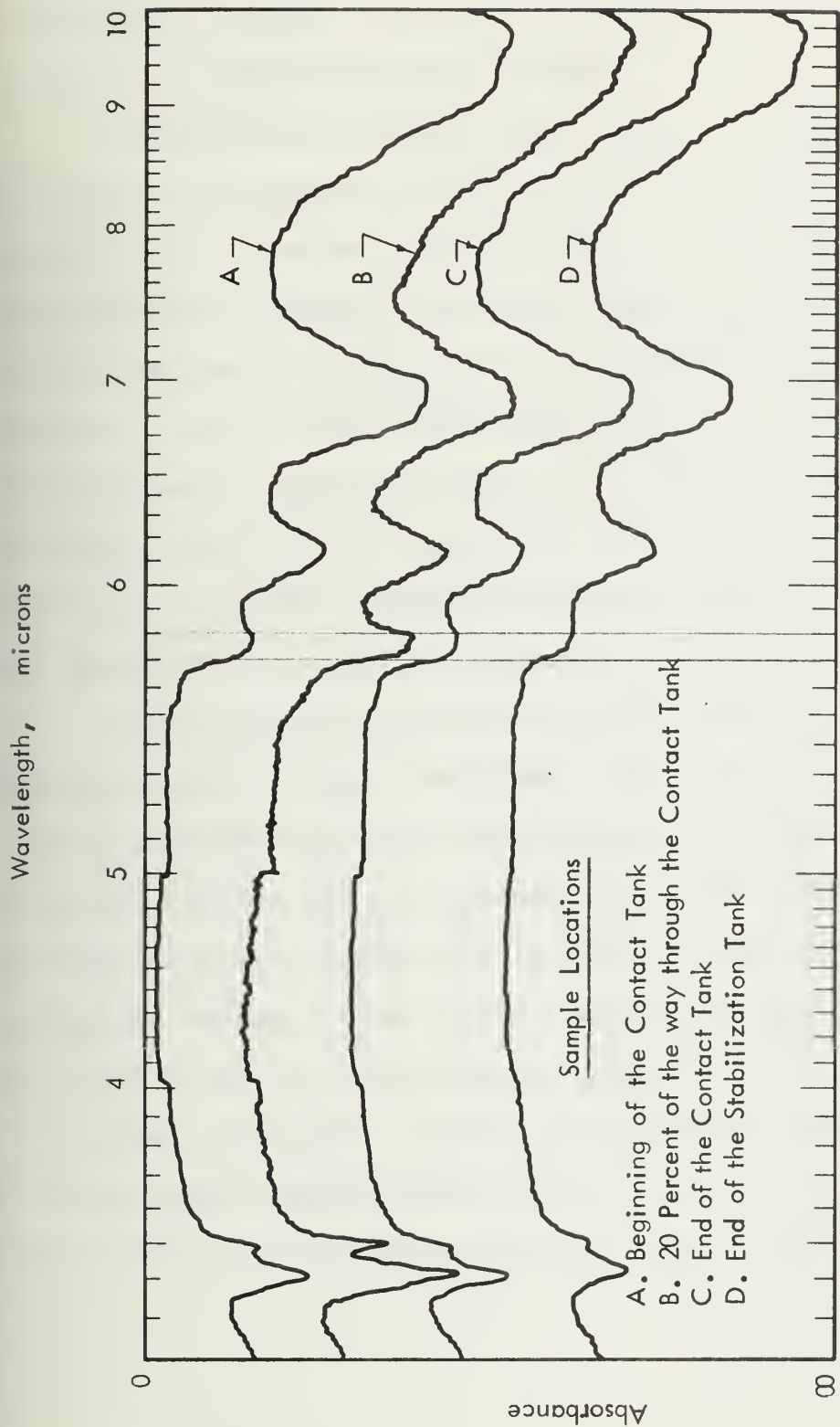
FIGURE 14   A COMPARISON OF THE INFRARED ABSORPTION SPECTRUMS OF ACTIVATED SLUDGE SAMPLES OBTAINED FROM DIFFERENT LOCATIONS IN THE URBANA-CHAMPAIGN MUNICIPAL WASTEWATER TREATMENT PLANT

suggest either the presence of a residual concentration of PHB in the sludge or the presence of some other material that has an absorbance at 5.8 microns. In order for PHB to be demonstrated, a significant rise and fall in absorbance should be observed.

In laboratory activated sludges, however, the peak was almost wholly associated with the PHB content of the sludge. Samples, obtained from a glucose-yeast extract sludge at various times after the sludge was fed, indicated that almost complete removal of the 5.8 micron peak could be obtained after long periods of endogenous metabolism. Figure 15 shows sludge samples harvested 1.5, 24, and 120 hours after feeding. The PHB content of the samples, as measured analytically, were 12.7, 0.6, and 0.1 per cent of the dry weight of the solids, respectively. Little or no absorbance was found 24 hours after feeding and only a slight absorbance 120 hours after feeding.

The influence of substrate on the absorption spectrum of laboratory sludges was also investigated. Sludges that were developed on various substrates throughout the course of this research were all started with seed from the stock glucose-yeast extract unit. The stock unit was known to contain PHB. Activated sludge from each of the new units was obtained 1.5 hours after feeding and washed with distilled water prior to running an I.R. analysis. These units were acclimated to the following substrates: glucose, sodium acetate, glutamic acid, and glucose-sodium acetate-caseamino acids. The I.R. spectrums of each sludge, as well as for the stock sludge, are shown in Figure 16. The
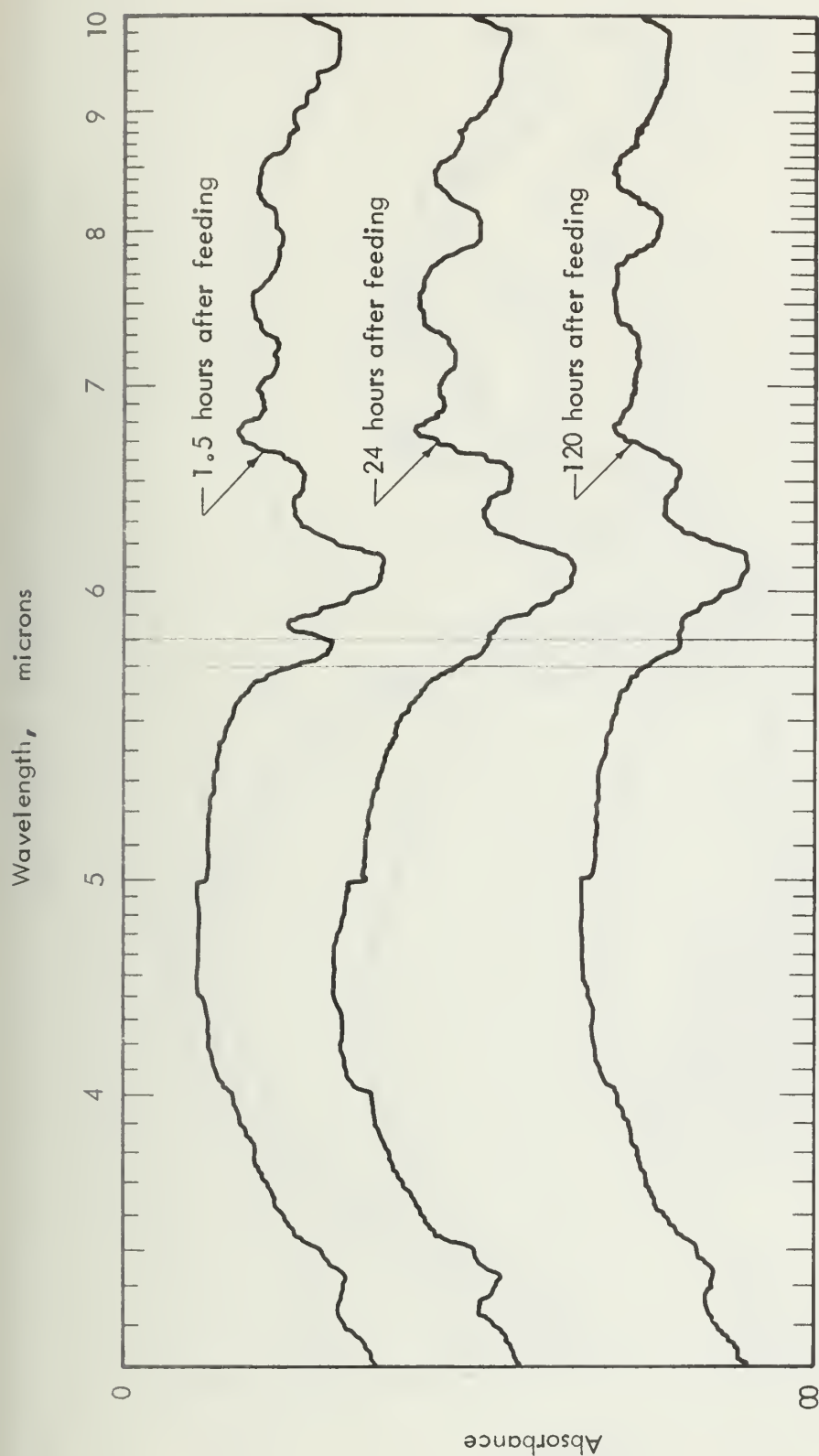
FIGURE 15 EFFECT OF SLUDGE AGE ON THE INFRARED ABSORPTION SPECTRUM OF LABORATORY ACTIVATED SLUDGE
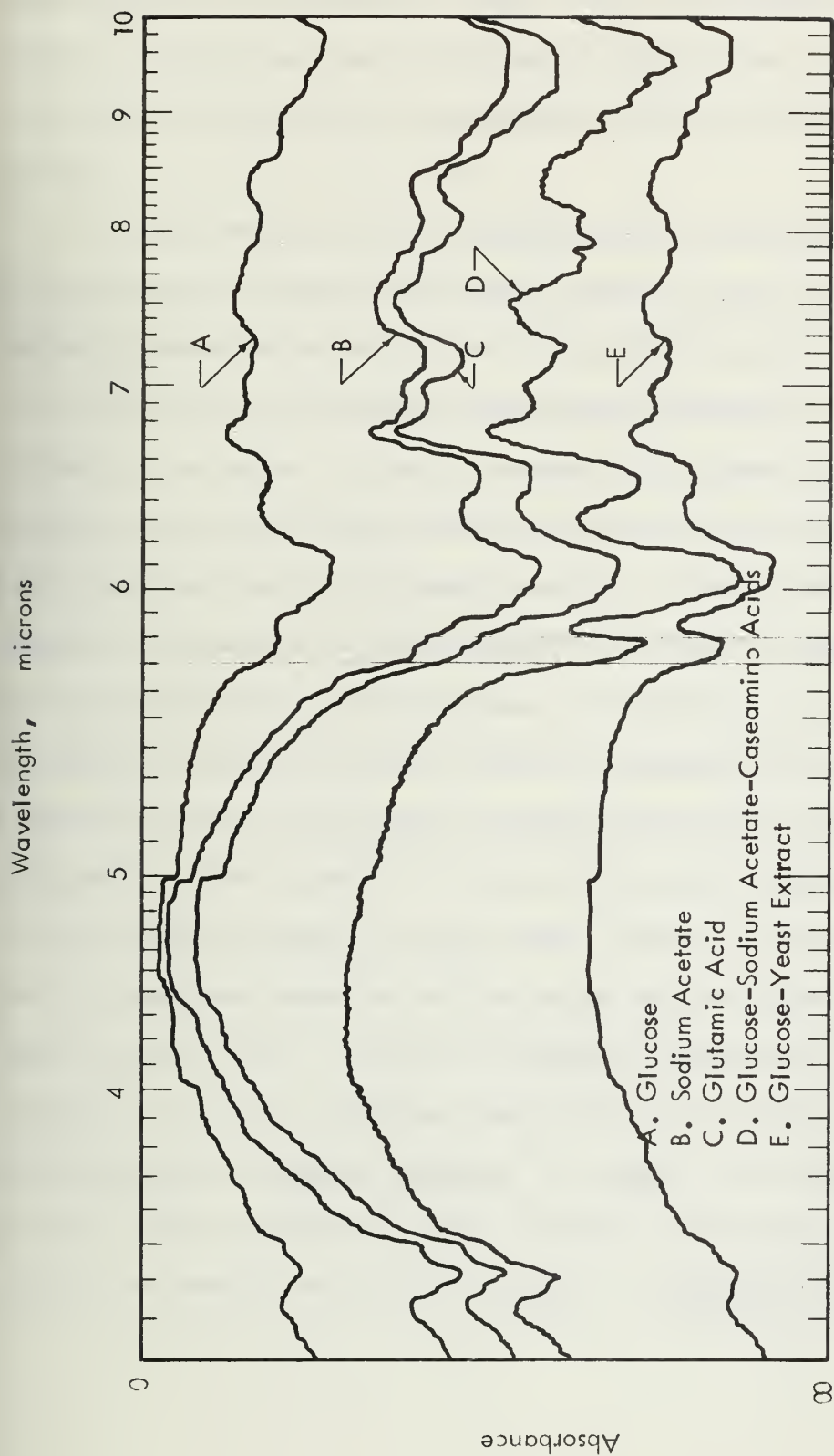
FIGURE 16 INFRARED ABSORPTION SPECTRUM OF ACTIVATED SLUDGE ACCLIMATED TO
DIFFERENT SUBSTRATES. SAMPLES HARVESTED 1.5 HOURS AFTER FEEDING

glucose, sodium acetate and glutamic acid sludges showed no absorbance characteristics representative of PHB. This was confirmed by analytical methods. The glucose-yeast extract and glucose-sodium acetate-caseamino acids sludges exhibited the characteristic 5.8 micron absorption band for PHB.

The governing factor for demonstrating the existence of PHB in the laboratory systems was probably related to the variety and species of organisms present in the five different units. The stock unit, from which all other units were seeded, was known to contain PHB synthesizing organisms. It may be assumed that a great variation of organisms was present in the stock unit because of the presence of an organic nitrogen source for protein synthesis. When a sample of these organisms was placed in a medium devoid of an organic nitrogen substrate, many could not grow and survive because of the strict requirement for the organic nitrogen substrate. Only the organisms that were able to synthesize protein from inorganic nitrogen were able to grow. Consequently, after a period of 10 days, the latter organisms were the only ones remaining from the initial seed. From I.R. data, it appears that the organisms washed out of the system because of the lack of organic nitrogen were, in fact, the organisms responsible for the synthesis of PHB. Two organisms that would fit this theory are Bacillus cereus and Zooglea ramigera. B. cereus is an organism that has been found in activated sludge, is known to synthesize PHB, and requires performed amino acids for growth. Z. ramigera has been shown

to contain PHB granules. It is not certain that this organism can survive long periods of growth in the absence of organic nitrogen. Dias and Bhat (48) found that not one of the 64 strains of Z. ramigera they isolated from activated sludge were able to grow without the presence of either vitamins or amino acids. McKinney and Weichlein (47) found that Z. ramigera, although isolated from activated sludge, could not flocculate in a glucose solution (void of nitrogen) after 48 hours, but when grown on sterile sewage it did flocculate. If flocculation is associated with growth, one would suspect that the organic nitrogen in sterile sewage was responsible for the growth of Z. ramigera. If it is indeed the case that Z. ramigera requires performed organic nitrogen for growth, then the organism would not be found in an acclimated glucose activated sludge system nor in any other unit in which organic nitrogen is not present. Thus by a process of selection, many organisms that synthesize PHB may not be present in laboratory-grown activated sludges.

b. Characterization of PHB

In order to further substantiate the presence of PHB, it was necessary to perform certain physical and chemical analyses on pure isolated PHB. PHB was isolated from 25 liters of activated sludge developed on a glucose-sodium acetate medium that was seeded initially with 100 ml of sludge from a laboratory unit which in turn was originally seeded with wastewater treatment plant activated sludge

and acclimated to glucose-sodium acetate and caseamino acids. Thirteen hours after feeding, all 25 liters were centrifuged at 3° C and the solids subjected to the PHB isolation procedure described in Chapter V. A white flaky precipitate formed, which, upon drying, turned slightly yellow. This material was used for the following analyses: melting point, carbon-hydrogen-oxygen content, COD, and infrared.

The melting point of three samples varied from 169° C to 172° C which is well within the range of values for PHB recorded by other investigators (49,61,62).

Carbon-Hydrogen-Oxygen analysis for one sample is shown in Table 7. The formula for the isolated PHB is $C_4H_6O_2$, which is precisely the formula for hydrolyzed poly-beta-hydroxybutyrate. The material submitted for analysis was 99.13 per cent pure.

The theoretical COD of a gram of PHB ($C_4H_6O_2$) is 1.67 gm. Four dry samples of PHB were weighed on an analytical balance and placed in COD flasks along with 20 ml of distilled water plus the remaining reagents for the COD test. The average COD of the four samples was 1.67 gm per gm of PHB.

TABLE 7

CARBON-HYDROGEN-OXYGEN ANALYSIS OF PHB
ISOLATED FROM LABORATORY ACTIVATED SLUDGE

Element	Per Cent by Weight	Per Cent Atomic Weight (Converted to C_4)
Carbon	56.32	4.00
Hydrogen	7.06	5.97
Oxygen	35.75	1.90

2. Glycogen

The presence of glycogen in a laboratory activated sludge unit was investigated by attempting to isolate glycogen from the TCA supernatant of activated sludge solids. The general procedure has been described by Gunsalus et al. (92). To a 1-liter aeration tank was added the following: 500 ml of mixed liquor from the stock unit, 2000 mg/l glucose, 500 mg/l yeast extract, 1000 mg/l ammonium sulfate, and minerals and buffer in accordance with Table 5. After 6 hours of aeration, 800 ml of mixed liquor was centrifuged and the solids washed with 0.03 molar phosphate buffer. The solids (670 mg) were homogenized with a Sorvall Omni-Mixer in 10 ml of cold 10 per cent TCA and placed at 4° C for one hour. At the end of this time the mixture was centrifuged and the supernatant added to 90 ml of 95 per cent ethanol. To the mixture was added a spatula tip of NaCl before placing it in a 50° C water bath for 1 hour. The precipitate was centrifuged and placed in a tared aluminum moisture dish. After drying at 100° C for 24 hours, the material was cooled and reweighed. Twenty-five milligrams of material were recovered which represented a glycogen contant of 3.5 per cent of the dry weight of MLSS. This was considerably less than the amount of lipid storage material found in the experiment in the previous section. Using the same substrates and seed organisms, 18.5 per cent of the solids were accounted for as PHB in the previous experiment.

It was decided to investigate further the occurrence of glycogen by developing activated sludge laboratory units on carbohydrate

substances only. Two activated sludge units, seeded with sludge from the stock unit, were acclimated to glucose-ammonium ion and starch-ammonium ion substrates, respectively. After a period of 10 days, experiments were conducted to determine the variation in the intracellular carbohydrates of the mixed liquor. It should be noted that intracellular carbohydrates include glycogen as well as the intermediate pools in the cell, and are measured by the carbohydrates present in the supernatant after treatment of the solids with 10 per cent TCA. The results are shown in Figures 17 and 18. The maximum amount of intracellular carbohydrates was 1.7 per cent of the solids in the glucose unit and 5.0 per cent of the solids in the starch unit. Both peaks occurred prior to the peak in MLSS.

It should be recalled from Chapter II that the definition of storage products did not stipulate a particular compound. It described the storage phenomena more on the basis of the rate of synthesis and degradation of a cellular constituent. Since the amount of cellular carbohydrates stored, as estimated by isolating glycogen and by measuring intracellular carbohydrates, was quite low (1 to 5 per cent of the solids), it was decided to investigate the use of total cell carbohydrates to describe the carbohydrate storage function. Observations on the rate of increase and decrease in total cell carbohydrates indicated that a storage function could be adequately estimated in this way. Figure 19 shows the results of an experiment using the stock unit sludge in which the total cell carbohydrate content increased from 18 to 39 per cent of the dry
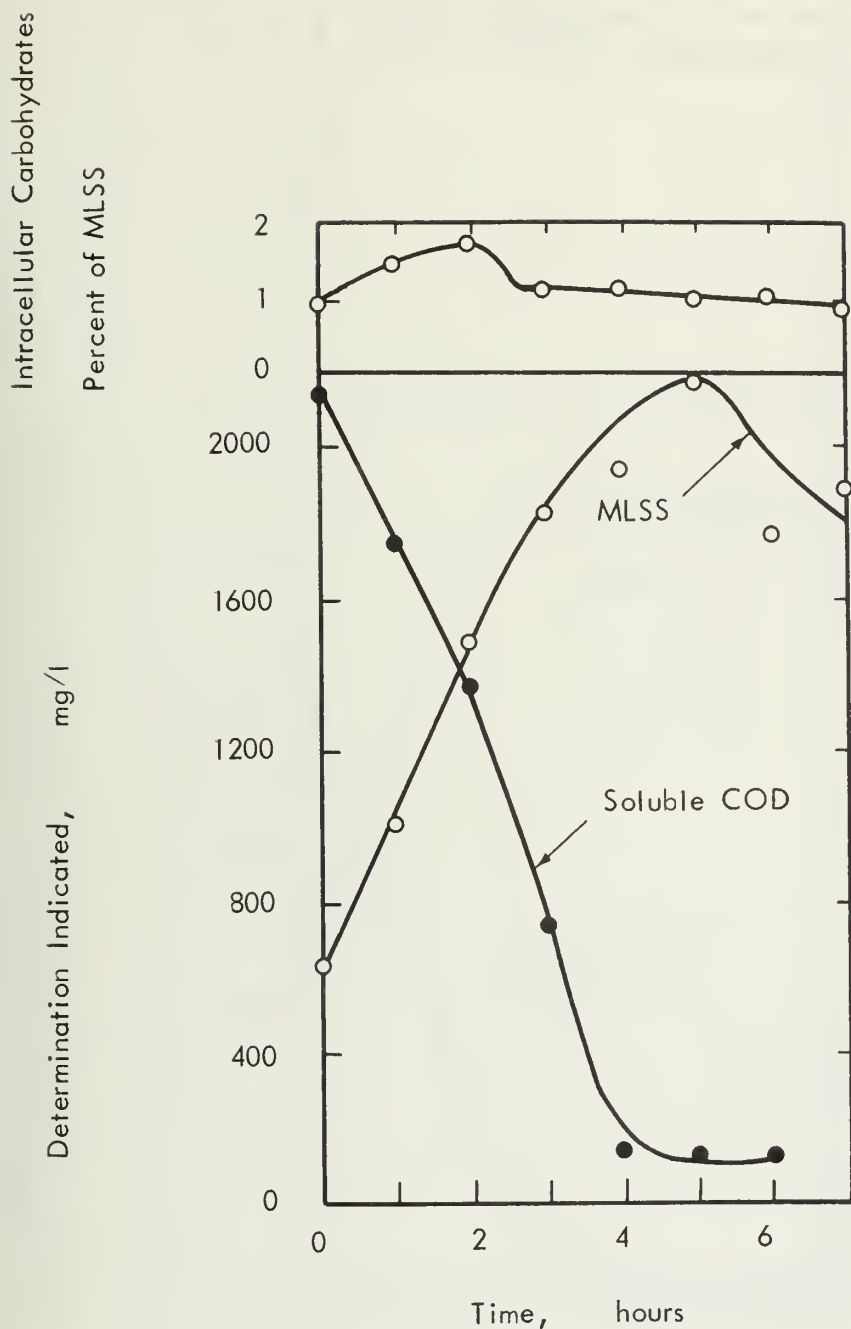
FIGURE 17   VARIATION IN INTRACELLULAR CARBOHYDRATE DURING
METABOLISM OF <u>GLUCOSE</u> BY AN ACCLIMATED ACTIVATED
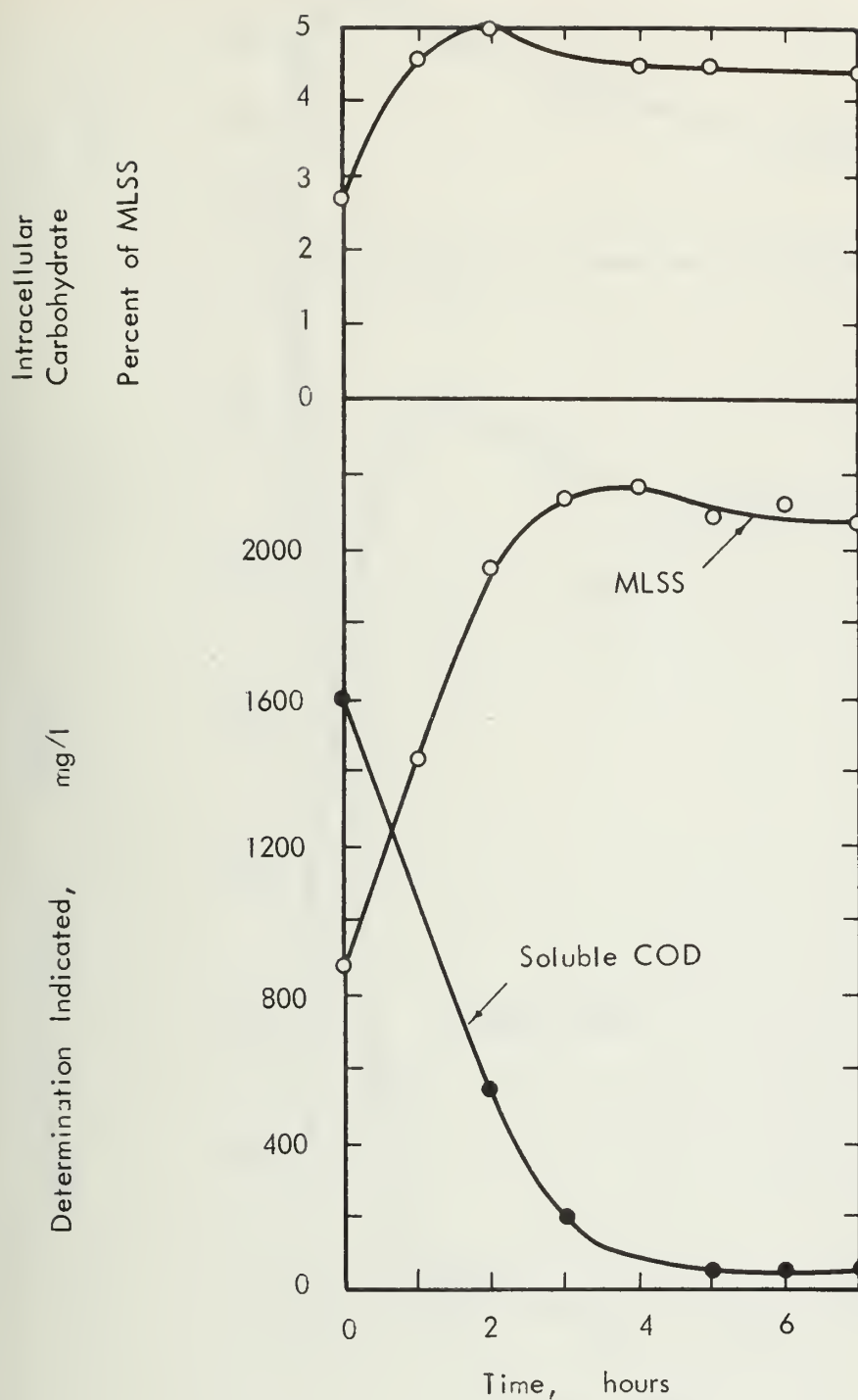SLUDGE

FIGURE 18 VARIATION IN INTRACELLULAR CARBOHYDRATE DURING
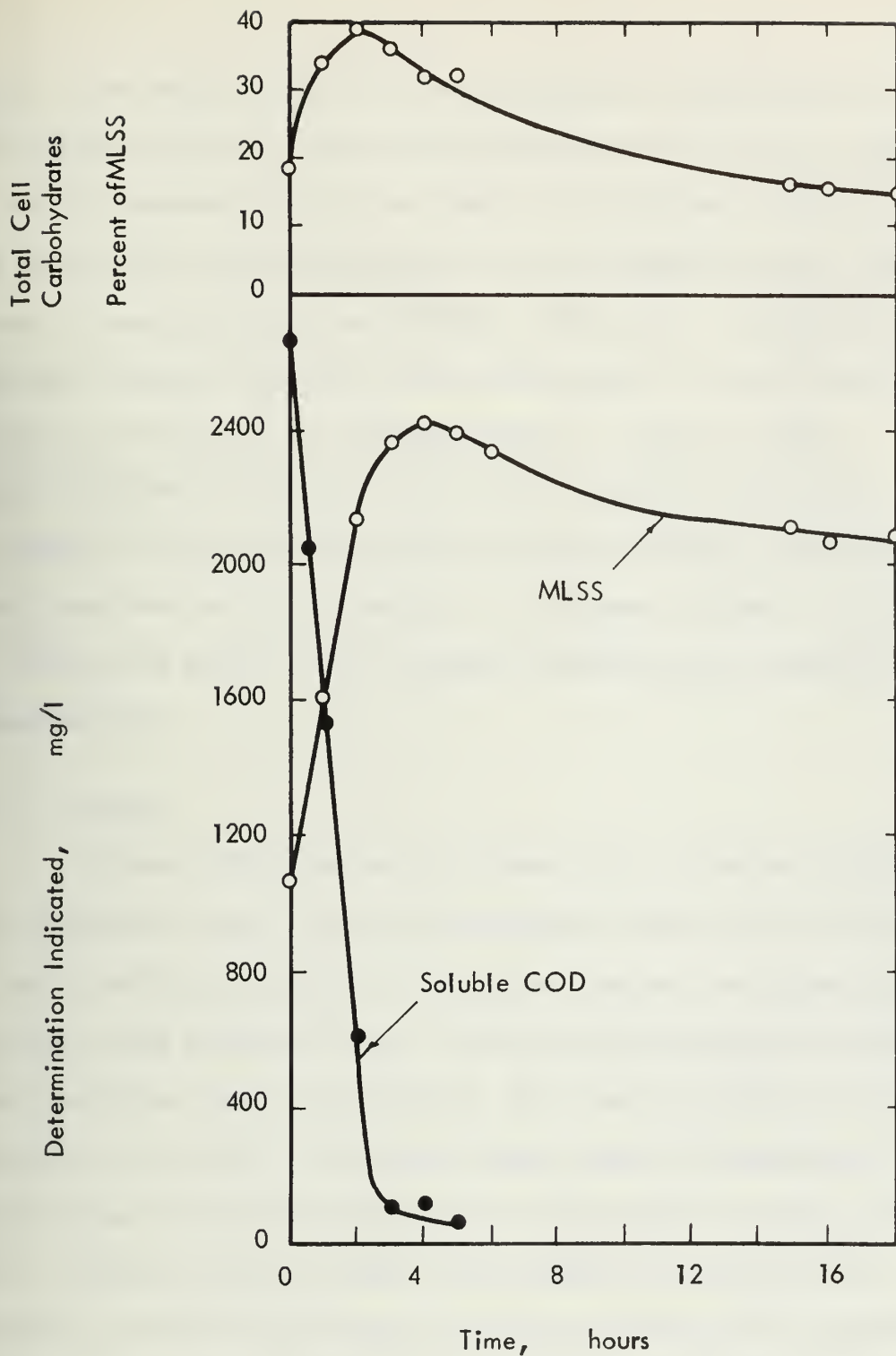METABOLISM OF STARCH BY AN ACCLIMATED ACTIVATED
SLUDGE

FIGURE 19 VARIATION IN THE TOTAL CELL CARBOHYDRATE DURING
METABOLISM OF GLUCOSE-YEAST EXTRACT BY ACTIVATED
SLUDGE FROM THE STOCK UNIT

weight of the solids during the first two hours of metabolism and subsequently decreased to the original value within the next 11 hours. The rate of synthesis and the net change was considered sufficiently high to use total cell carbohydrates for the estimation of the carbohydrate storage function in an activated sludge unit. It should be remembered, however, that all of the carbohydrates in a cell cannot be used as a measure of the storage capacity. There is a basic amount of carbohydrate which is part of the integrity of the cell. This latter portion, sometimes referred to as structural carbohydrates, is not considered as storage material. No attempt was made to identify the carbohydrate material that was being synthesized and classified as storage material.

3. Summary

Although PHB could not be isolated from wastewater treatment plant activated sludge, there was very strong evidence of its existence. Infrared absorption spectrums of the sludge obtained from three municipal activated sludge treatment plants in Illinois demonstrated the existence of the absorption peak at 5.8 microns. This peak is typical of the absorption of pure PHB. Laboratory sludges, seeded with municipal treatment plant activated sludge and fed glucose plus an organic nitrogen source, definitely contained PHB. The polymer was isolated from a laboratory unit and characterized according to melting point, carbon-hydrogen-oxygen content, COD, and I.R. analysis. All findings were in agreement with theoretical values and with those values found in the literature.

The intracellular carbohydrate content of laboratory activated sludges did not provide an adequate estimation of the amount of carbohydrate storage. The measurement of net change in total cell carbohydrates, on the other hand, fitted the definition given in Chapter II and was, therefore, adopted for use in describing the carbohydrate storage function.

B. Parameters Influencing the Synthesis of Storage Products

The subject matter deals primarily with the parameters that influence the synthesis of storage material in an activated sludge system. However, during initial considerations, thought was given to complementing the carbohydrate and PHB test with other analytical tests which would provide a clearer insight into the variability of the composition of the mixed liquor solids. The Folin protein test as well as the COD of the solids were, therefore, incorporated in hopes they could serve as an adjunct in data evaluation.

By including the measurement of protein, it was possible to obtain a solids balance of cellular carbohydrates, protein and PHB against the total measured solids of the mixed liquor (MLSS). These three fractions represent the major divisions of organic matter in the cell. In all cases there was a remaining fraction, which is referred to as "other" material. Similarly, a balance could be made on the basis of COD, since the measurement of solids-COD was made a part of the routine testing. For this type of balance, the weight of carbohydrates, protein and PHB were converted to their COD values by

appropriate factors that were determined analytically. The carbohydrate standard was glucose, and therefore, all carbohydrate weights were multiplied by the experimentally determined value of 1.045 gms COD/gm of glucose. In the protein test, the standard was bovine serum albumin which, experimentally determined, contained 1.43 gms.COD/gm of protein. The value 1.42 has been experimentally determined for casein (26). PHB solids were similarly converted by using the factor 1.67 gms COD/gm PHB. When the three constituent COD's were added together and their total subtracted from the solids-COD there was always a remainder which is referred to as "other."

In order to maintain continuity, the data are discussed immediately after its presentation and a separate chapter is devoted to a summary discussion.

1. Food to Microorganism Ratio

One of the primary criteria in the design of activated sludge systems is the loading rate, or as frequently referred to, the food to microorganism (F/M) ratio. It is the ratio of the daily weight of organic substrate that is supplied to the activated sludge aeration tank divided by the total weight of the sludge solids under aeration. In the laboratory fill and draw units used in these studies, the F/M ratio was determined as the total COD in the soluble feed divided by the weight of the mixed liquor suspended solids present at time zero. Since the F/M ratio is an important factor in the design and control of the activated sludge process, its influence on the synthesis of storage products was the initial area of investigation.

Four 2-liter fill and draw units were seeded with mixed liquor from the stock glucose-yeast extract unit and acclimated to different F/M ratios according to the schedule shown in Table 8. The F/M ratios shown in Table 8 are theoretical ones that were calculated on the basis of 50 per cent synthesis in all units. The actual experiment F/M ratios are the ones shown in Figures 20 and 21. It was considered necessary to acclimate the activated sludge units to different F/M ratios rather than taking one sample of mixed liquor and adjusting it to a one-pass F/M ratio for which it was not acclimated. The latter type of operation was considered as a quantitative shock loading which does not adequately describe the day-to-day operation of a wastewater treatment plant.

TABLE 8

DAILY SCHEDULE OF OPERATION FOR UNITS ACCLIMATED
TO DIFFERENT F/M RATIOS

	Unit 1	Unit 2	Unit 3	Unit 4
Per Cent Wasting	20	33	50	75
F/M Ratio*	0.5	1.0	2.0	6.0
Substrate Concentration as mg/l COD				
Glucose	500	500	1000	1000
Yeast Extract	500	500	1000	1000
$(NH_4)_2SO_4$, (mg/l)	500	500	1000	1000
COD/N Ratio	6.8	6.8	6.8	6.8

*Theoretical F/M ratio based on 50 per cent synthesis.

All units were acclimated for a period of not less than 10 days before the experiment was conducted. During this time all the units developed good settling characteristics. Each system was supplied a

nutritionally balanced feed in which the COD/N ratio was 6.8:1.

Minerals were supplied in accordance with Table 5. The experimental

procedure and sampling protocol is described in Chapter V.

The four experiments were carried out at F/M ratios of

0.78, 1.52, 4.30, and 7.25. The results of each experiment are shown

in Figures 20 and 21. The general nature of the response to

substrate was an increase in the concentration of all the measured

constituents as the substrate COD was removed from solution. The

units that were operated at F/M ratios of 0.78, 1.52, and 4.30

exhibited an immediate increase in biological solids. The highest

F/M ratio unit underwent a slight lag typical of the familiar bacterial

growth curve.

There were a number of ways considered for demonstrating the

carbohydrate and PHB storage function. However, the primary concern

was to calculate the carbohydrate increase per cell while the number of

cells was increasing. Since the solids weight is not an accurate

measure of the number of cells, it was decided to use protein for the

purpose. Accordingly, the carbohydrate concentration was divided by

the protein concentration and this value was plotted against time. This

provided a reasonably accurate determination of the carbohydrate

variation per cell during metabolism. The use of protein for the

quantitative estimation of cell count is an accepted procedure and, as

will be shown later, it also constitutes a better criterion for

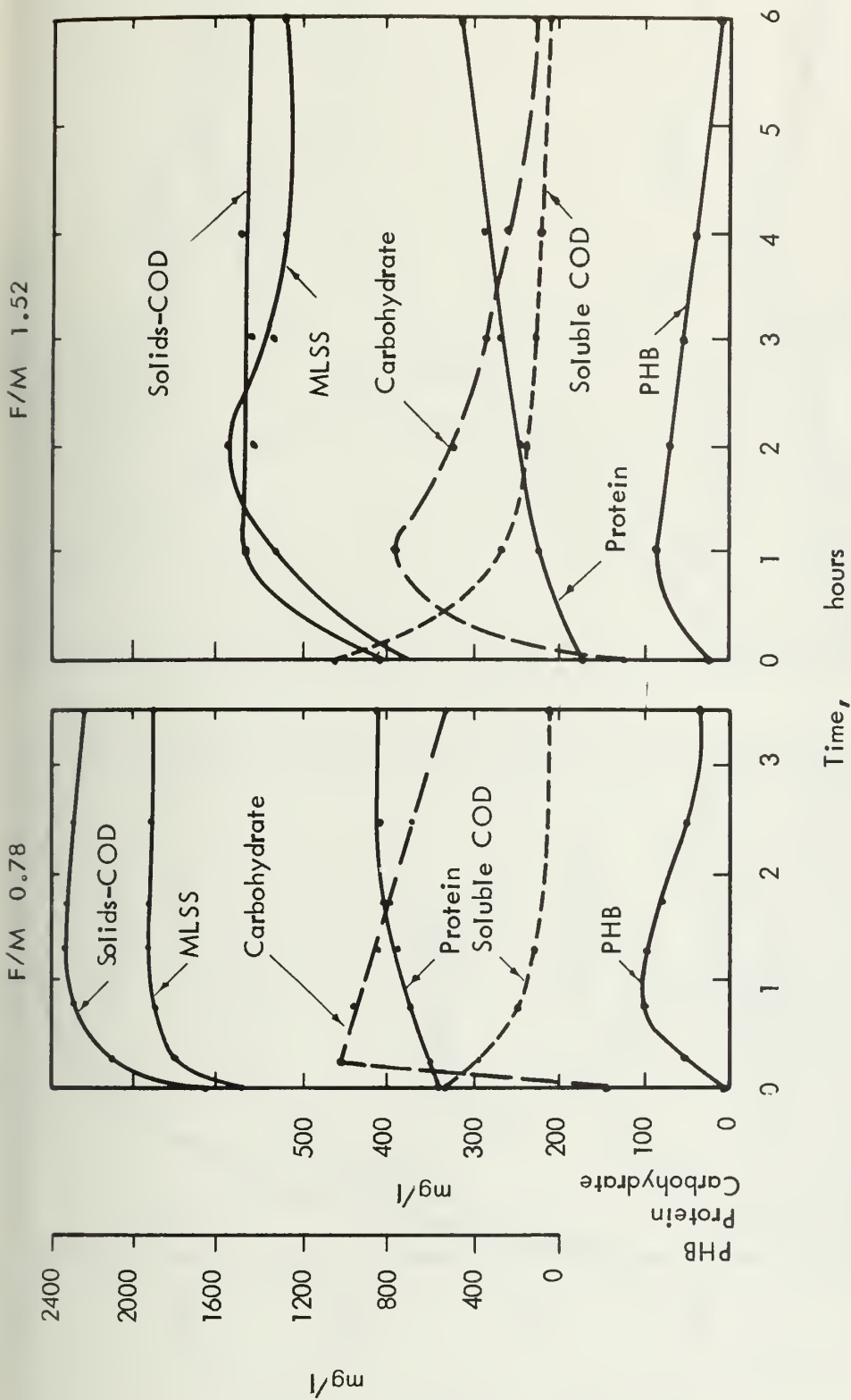describing the removal of substrate than the use of mixed liquor

FIGURE 20 SYSTEM RESPONSE OF GLUCOSE-YEAST EXTRACT ACTIVATED SLUDGE UNITS
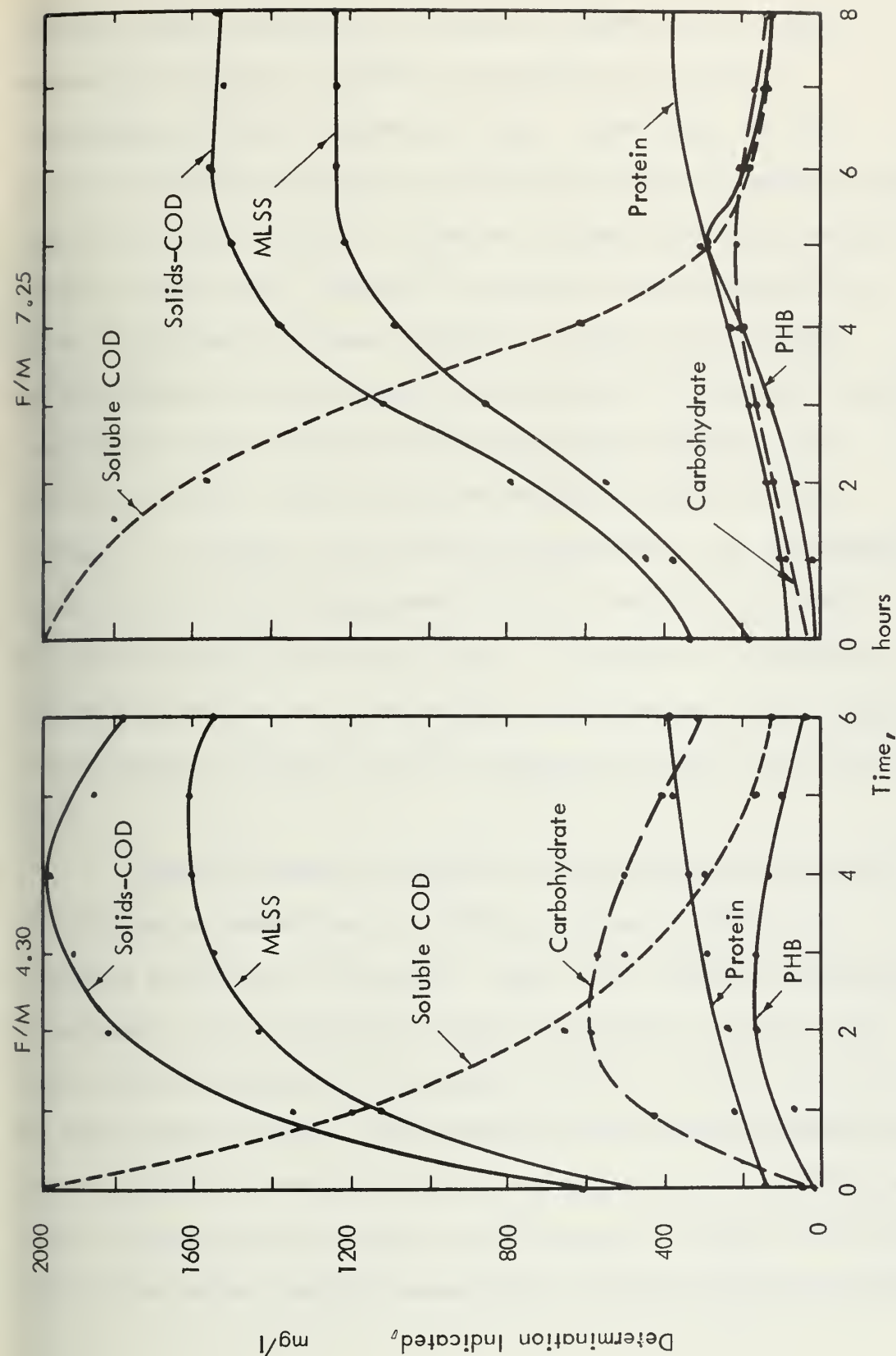ACCLIMATED TO F/M RATIOS OF 0.78 AND 1.52

FIGURE 21   SYSTEM RESPONSE OF GLUCOSE-YEAST EXTRACT ACTIVATED SLUDGE UNITS
ACCLIMATED TO F/M RATIOS OF 4.30 AND 7.25

suspended solids concentration. Holme and Palmstierna (93) have
measured the cell count, protein concentration and glycogen
concentration in batch cultures of E. coli. They found that the
quantity of protein-nitrogen per cell did not vary significantly under
conditions of either carbon or nitrogen starvation and was directly
related to cell count. However, the quantity of glycogen per cell
varied 10-fold under the same conditions. Their results indicate
the reliability of using protein as a measure of cell number. It was
interesting to note that when the storage product, glycogen, was
plotted as mg/cell. Using the latter method, the data were more
meaningful. Therefore, in the following experiments, the carbohydrate,
as well as the lipid storage material will be expressed in terms of
the protein content of the mixed liquor. It should be noted that on
the basis of total mixed liquor weight, the curves were quite similar
to those shown in Figures 22 and 23, especially for the three low F/M
ratios.

Figure 22 shows the variation in the carbohydrate content of
the cells during metabolism of substrate by organisms that were
acclimated to different F/M ratios. There was an immediate high rate
of increase in the carbohydrate content of the cells from all units
except the one operating at the highest F/M ratio. Within two hours,
the three units operating at the lower F/M ratios had synthesized the
maximum quantity of carbohydrates. The disappearance of carbohydrates
in the cell was also quite rapid in all the units. Within 5 hours after
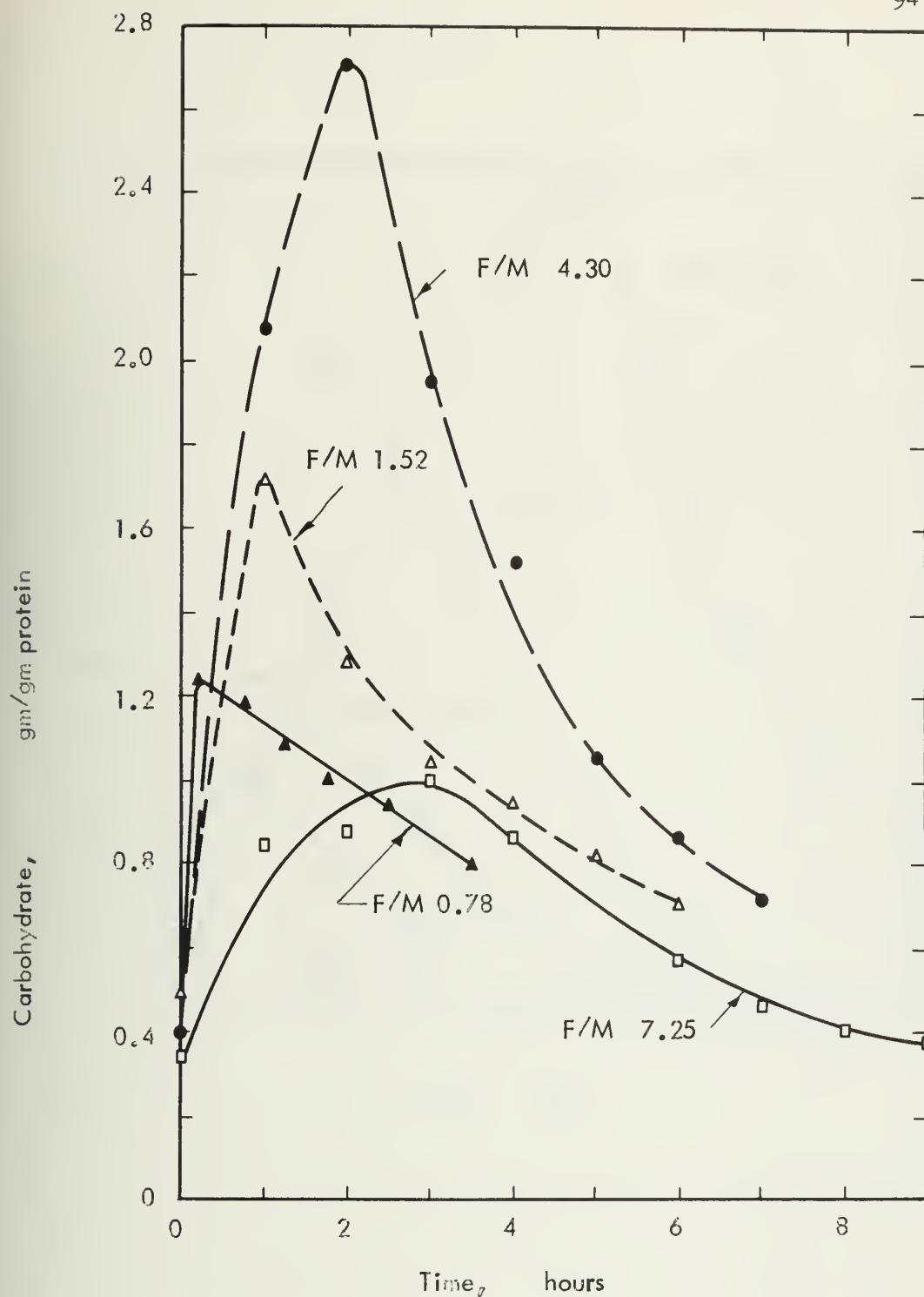reaching the maximum carbohydrate content, the cells from all units had

FIGURE 22   VARIATION IN CELLULAR CARBOHYDRATE DURING METABOLISM
OF SUBSTRATE BY ACTIVATED SLUDGE ACCLIMATED TO
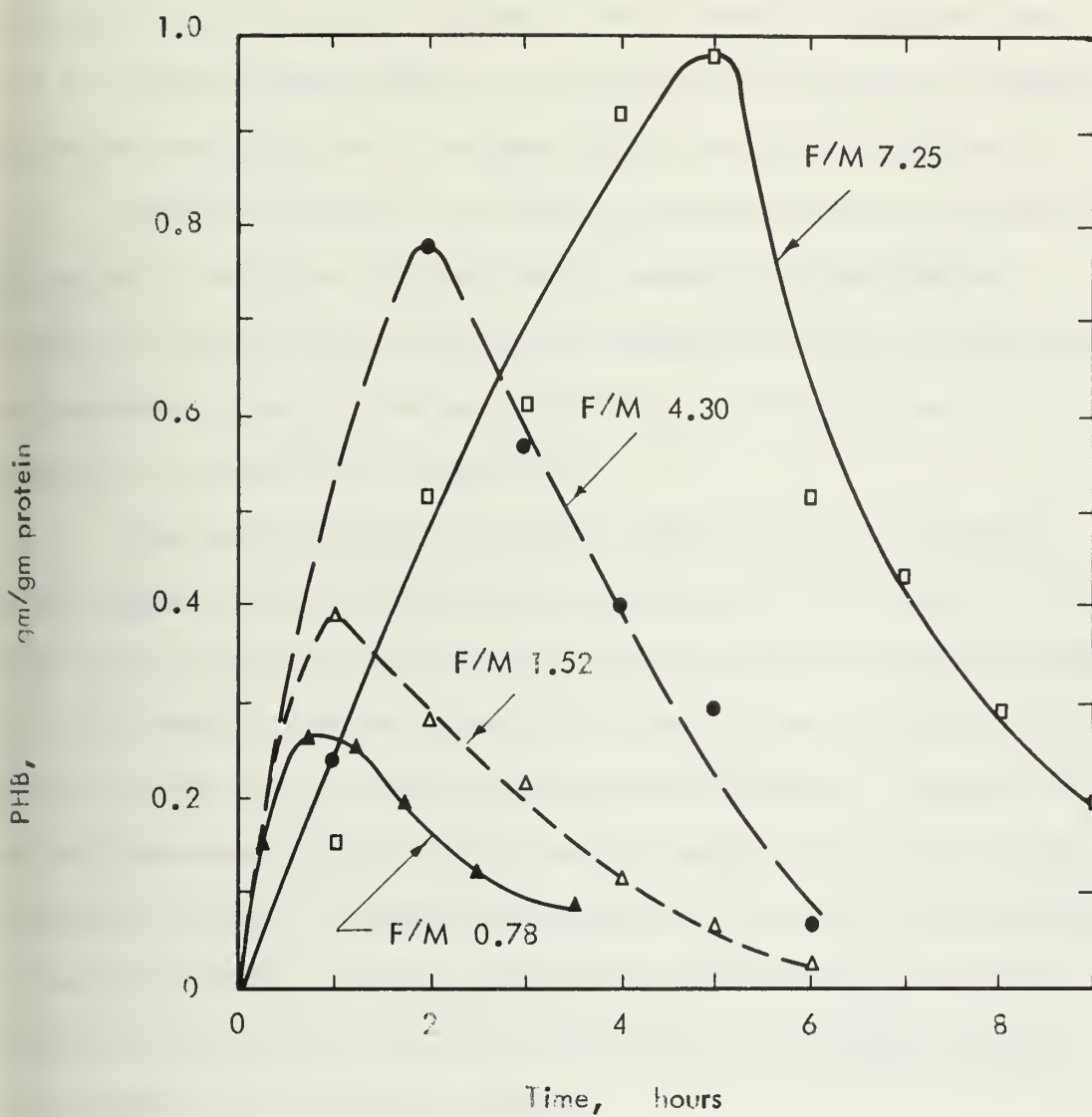DIFFERENT F/M RATIOS

FIGURE 23   VARIATION IN CELLULAR PHB DURING METABOLISM OF
SUBSTRATE BY ACTIVATED SLUDGE ACCLIMATED TO DIFFERENT
F/M RATIOS

depleted over 90 per cent of the reserve material. The initial rate of degradation appeared to be directly proportional to the F/M ratio below F/M 7.25. This rapid synthesis and degradation indicated that much of the total carbohydrates of the cell were truly storage products and fitted the definition of storage products as given in Chapter II.

The PHB variation in the cells from the four units, as shown in Figure 23, exhibited the same rapid synthesis and degradation pattern for the three lower F/M ratio systems as was described for cell carbohydrates. However, the total quantity of PHB stored was a maximum in the highest F/M ratio unit.

The maximum amount of storage products that the activated sludge systems could accumulate was influenced by the F/M ratio. This relationship is shown in Figure 24, which represents the maximum values from each curve in Figures 22 and 23. It can be seen that more PHB is stored per cell as the loading on the system increases. However, for carbohydrate storage there appears to be a maximum amount that can be accumulated per cell. Between F/M ratios of 0.78 and 4.30 the maximum carbohydrate storage increased with increasing F/M ratio. When the loading was increased to F/M 7.25, the ability of the cells to store carbohydrates was considerably reduced.

The amount of substrate that is channeled into storage material was also calculated. On the basis of substrate COD removed from solution and the increase in carbohydrate-COD and PHB-COD it was possible to determine the maximum per cent of substrate that was converted to these
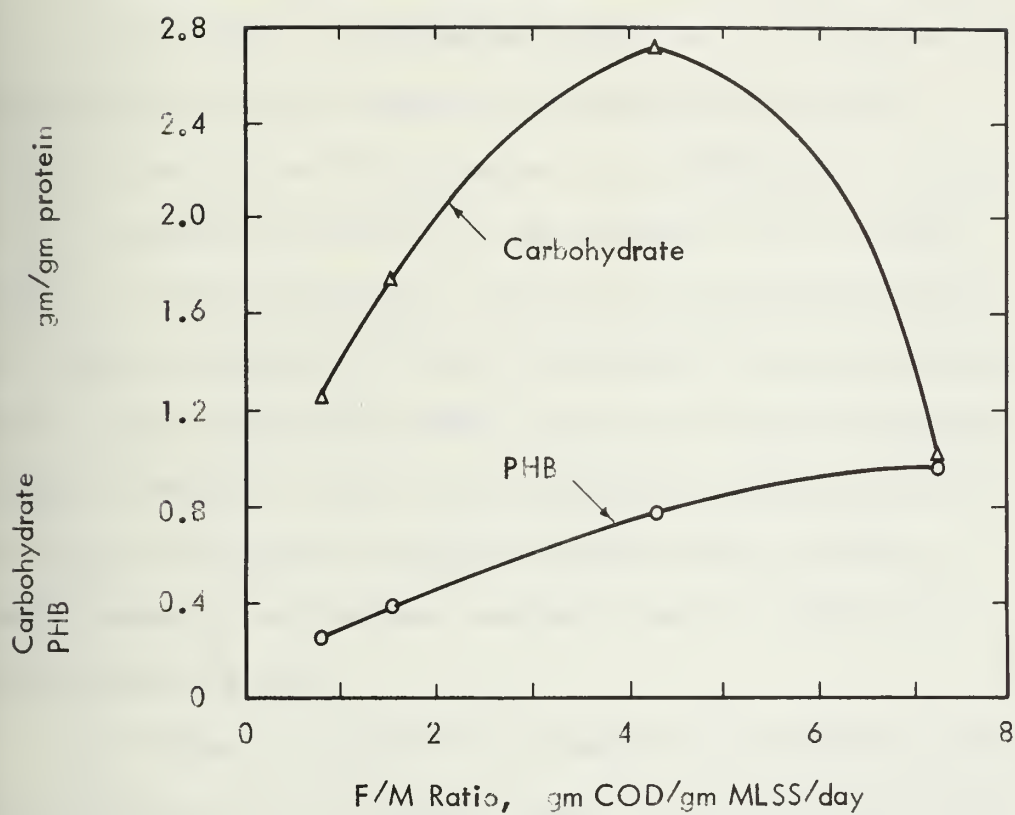
FIGURE 24   INFLUENCE OF F/M RATIO ON THE MAXIMUM AMOUNT
OF CELLULAR CARBOHYDRATE AND PHB STORED

storage products. In order to reduce the amount of error introduced

as a result of the synthesis of one storage product from the degradation

of another, the balance was calculated when the carbohydrate concentra-

tion first reached its maximum value. The results, shown in Figure 25,

indicate that cells acclimated to F/M ratios between 0.78 and 4.30

convert approximately 40 per cent of the substrate to carbohydrates

and 20 per cent to PHB. At F/M ratios higher than 4.30, the percentage

of substrate used for the synthesis of carbohydrates decreases.

Because there was such an abrupt change in the carbohydrate synthesis

of the unit operated at F/M 7.25, it was decided to set up another

experiment to verify this value. The new unit was operated similar

to the other four and after 10 days of acclimation, an experiment was

conducted. Cell carbohydrates, MLSS, and soluble COD were measured.

The amount of substrate used for the synthesis of carbohydrates at

F/M 6.11 was in accordance with the other data which indicated a

decreasing amount of carbohydrate synthesis when the loading was

increased beyond F/M 4.30.

Also shown in Figure 25 is the amount of substrate respired.

It was calculated as the difference between the substrate COD removed

and the increase in solids - COD.

Calculations of the amount of substrate used for protein

synthesis indicated that systems operated at F/M ratios greater than

4.30 were more adapted, physiologically, for growth and replication

than for the storage of substrate. At F/M 4.30, 8.6 per cent of the
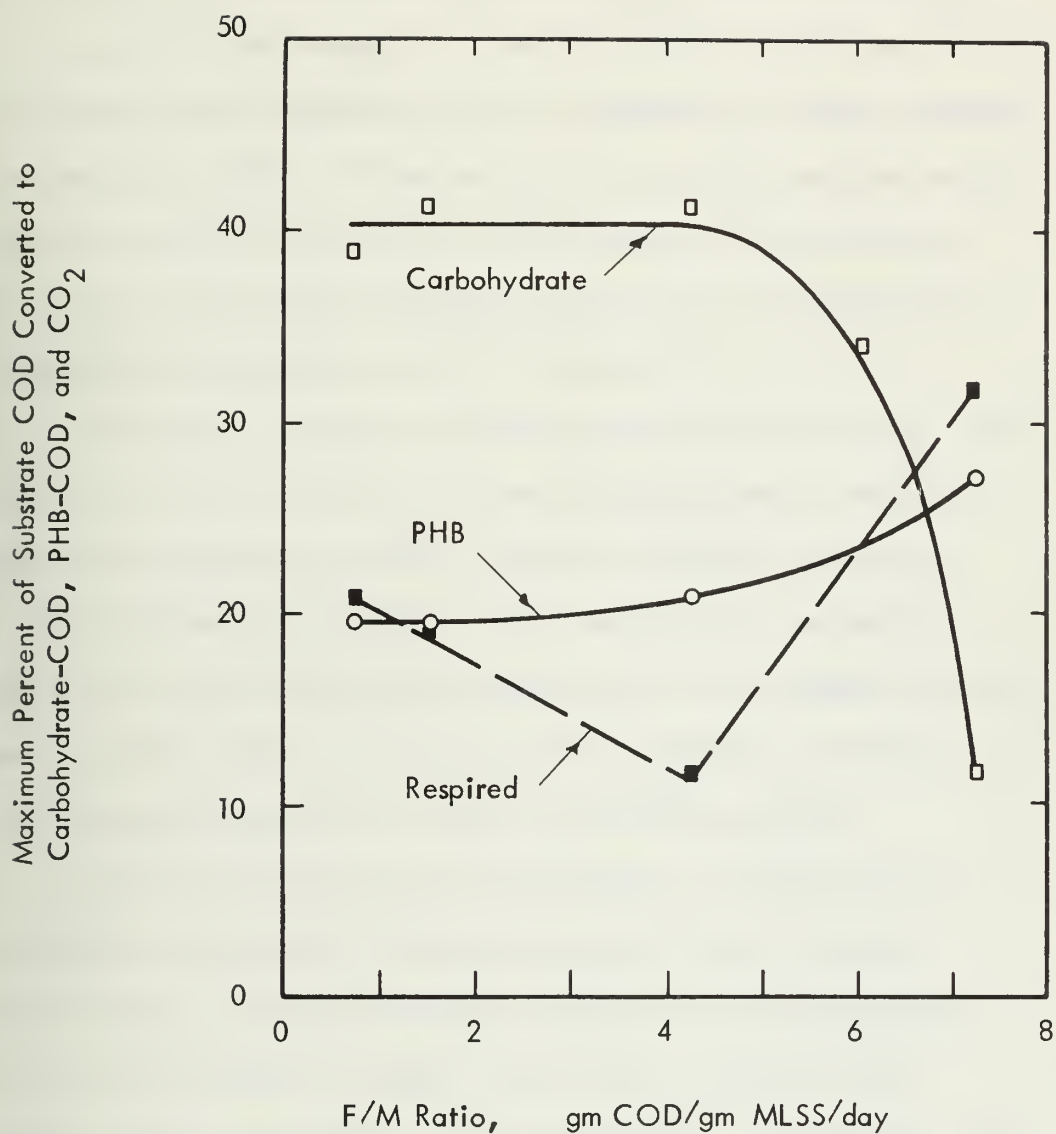
FIGURE 25   INFLUENCE OF F/M RATIO ON THE DISTRIBUTION OF SUBSTRATE
INTO CARBOHYDRATE AND PHB STORAGE PRODUCTS AND
RESPIRATION

substrate COD was used for protein synthesis, whereas, at F/M 7.25, 16.2 per cent of the substrate COD was used for protein synthesis.

At F/M ratios between 0.78 and 4.30 more than 60 per cent of the substrate COD was channeled into the synthesis of storage products (carbohydrates plus PHB). If the amount of substrate respired is also considered, more than 80 per cent of the substrate removed can be accounted for in these three metabolic mechanisms. The importance of these mechanisms, therefore, cannot be overlooked.

In summary, it can be said that increasing the loading rate up to an F/M ratio of 4.30 in an activated sludge process results in a sludge that accumulates a greater amount of storage products per cell. At an F/M ratio of 0.78, the cells were able to accumulate a maximum of 1.25 gm carbohydrate/gm protein and 0.26 gm PHB/gm protein whereas, at an F/M ratio of 4.30 the cells accumulated a maximum of 2.71 gm carbohydrate/gm protein and 0.77 gm PHB/gm protein.

An activated sludge system operating at an F/M ratio of 4.30 represents the optimum loading rate that achieves the most economical design. At this F/M ratio, the mixed liquor organisms require the least amount of oxygen while at the same time they contain the highest percentage of stored material. Furthermore, the rate of degradation of stored material in the F/M 4.30 unit was more rapid than in the lower F/M units.

When the loading rate was increased beyond the optimum F/M 4.30, there was a considerable reduction in storage product synthesis as well as an increase in oxygen requirement.

There appeared to be a distinct difference between the physiological condition of the cells in the F/M 7.25 unit and the cells in all the other units. In the three lower F/M units, the microbial cells were adapted to a metabolic response that stressed the storage of substrate, whereas in the highest F/M ratio unit, the cells were acclimated to a metabolic response that precluded storage of substrate in favor of protein synthesis. This was not a reflection of any nitrogen deficiency in the lower F/M units but rather of the environmental conditions under which the cells were acclimated. As the F/M ratio increased, the time required to remove a given percentage of the substrate lengthened and the period of stabilization therefore decreased. Since the stabilization period is a dynamic state of metabolism in which anabolic reactions may take place (97,98), any shortening of this period could result in the loss of certain enzymes and enzyme systems. This explanation could account for the reduced storage function found in the F/M 7.25 unit.

The higher amount of substrate respired also offers proof that protein synthesis was stressed in the F/M 7.25 unit. The synthesis of a protein molecule requires more than 10 times the amount of energy that polysaccharide synthesis requires and almost twice as much as that required for lipid synthesis (94). As judged by the amount of CO_2 respired, more energy was required during the metabolism of substrate in the F/M 7.25 unit than in any of the other units.

The activated sludge process is generally not operated at loading rates as high as F/M 7.25. According to the data presented here, it would not be economical to load a system that high. There is, however, a modification known as dispersed aeration (96) which has been used in practice. This method of waste treatment involves the aeration of wastes with a much lower concentration of organisms than would be used in any of the other activated sludge modifications. Typically, the process results in a high amount of substrate being respired and long periods of aeration for the removal of substrate. The response is similar to that observed in the F/M 7.25 units.

2. COD to Nitrogen Ratio

The purpose of this study was to determine the influence of nitrogen deficiency on the synthesis of storage products.

Three 2-liter fill and draw units were acclimated for a period of not less than 10 days to COD/N ratios of 16.5, 31.4, and 56.0. The nitrogen content was based on total yeast extract nitrogen plus ammonium sulfate nitrogen. All units were operated on a 50 per cent mixed liquor wasting schedule and were seeded with sludge from the stock glucose-yeast extract unit. The concentration of substrates and ammonium sulfate used in the feed are shown in Table 9. Both glucose and yeast extract had been shown previously (Figure 12) to be removed from solution concurrently. Therefore, varying the concentration of the two substrates would not be expected to alter their removal characteristics by the activated sludge used in this experiment.

Appropriate quantities of mineral salts and buffer were added (Table 5) along with tap water. Periodic measurements of pH on the three units ranged from 7.0 to 8.0. Every other day the sludge settling volume was measured in a 100-ml cylinder. The settled sludge never exceeded a volume of 10 ml for any of the units. The zero hour solids concentration of all three units was near 900 mg/l. The experimental procedure and sampling protocal is described in Chapter V.

TABLE 9

SUBSTRATE FEED CONCENTRATIONS FOR THE OPERATION
OF ACTIVATED SLUDGE UNITS ACCLIMATED
TO DIFFERENT COD/N RATIOS

COD/N Ratio	16.5	31.4	56.0
Glucose-COD, mg/l	522	1567	2090
Yeast Extract-COD, mg/l	2000	1000	500
$(NH_4)_2SO_4$, mg/l	50	50	50

Figure 26 shows the system response of the three activated sludges that were acclimated to different concentrations of nitrogen. The initial solids concentration and the initial substrate COD for all units were similar. Therefore, each unit had the same F/M ratio, 2.83.

Substrate removal rate per gram of solids varied considerably between units during the first 2 hours of each experiment. However, on the basis of protein weight, the removal rate was quite comparable. The comparison is shown in Table 10. Based on the solids content, the rate of substrate removal varied from 0.48 to 1.13 COD removed/(hr) (gm MLSS), while based on the protein content of the mixed liquor, the
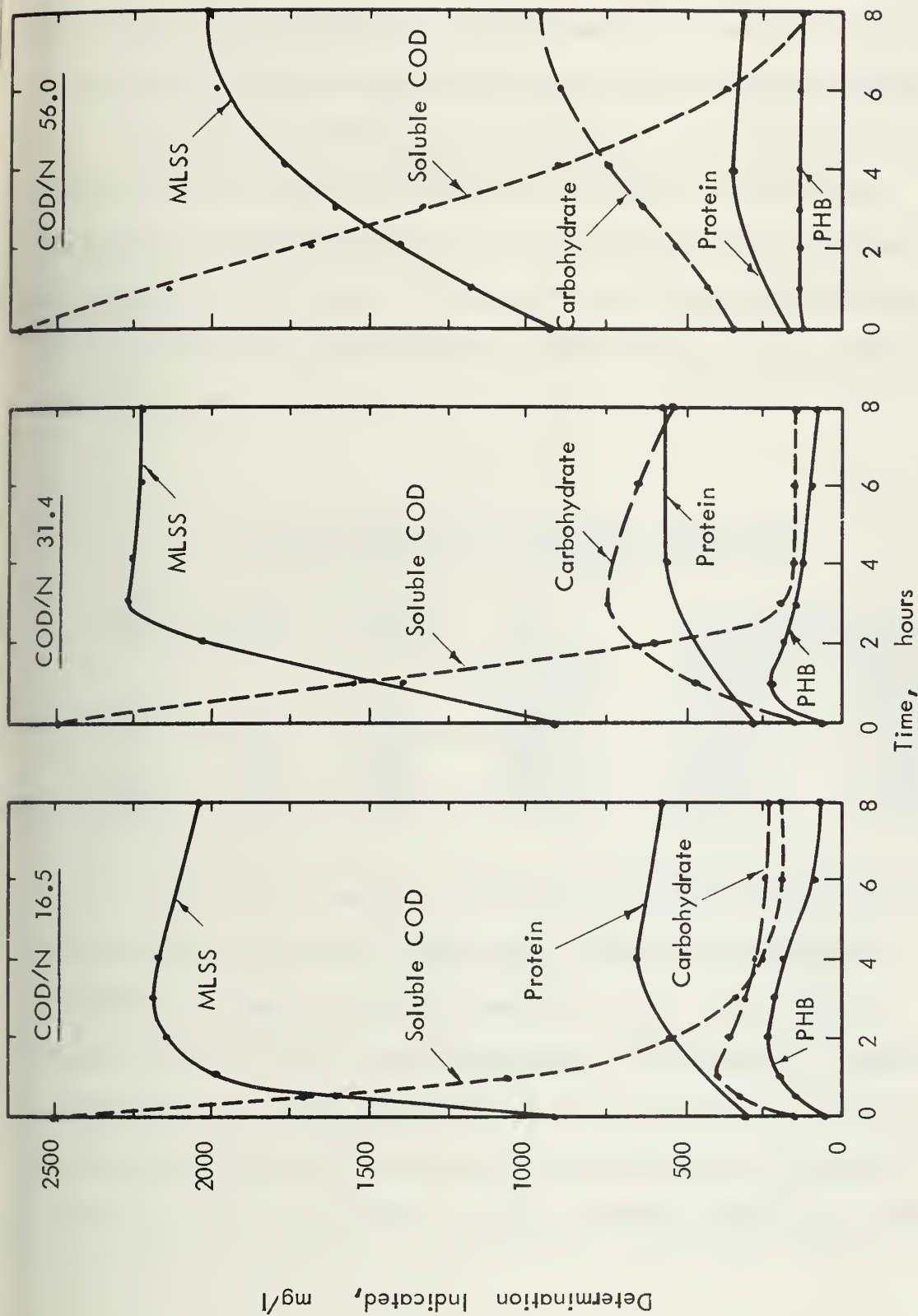
FIGURE 26   SYSTEM RESPONSE OF GLUCOSE-YEAST EXTRACT ACTIVATED SLUDGE ACCLIMATED TO DIFFERENT COD/N RATIOS

rate of substrate removal varied from 2.85 to 3.45 gm COD removed/(hr) (gm protein). As expressed on a solids basis, the increase in rate was 136 per cent, whereas, expressed as protein, the increase in rate was only 21 per cent. It would appear, therefore, that the systems were similar in their capacity for removing substrate, the difference being that at the high COD/N ratio, there were actually less organisms per gram of solids. The excess in weight per organism was attributed to a greater amount of non-degradable carbohydrates such as capsular polysaccharides.

TABLE 10

RATE OF COD REMOVAL FOR ACTIVATED SLUDGE UNITS
ACCLIMATED TO DIFFERENT COD/N RATIOS

COD/N	Initial Solids	Initial Protein	COD Removed in Initial 2 Hours	Rate of COD Removal in Initial 2 Hours	
				gm/gm of solids	gm/gm of protein
	mg/l	mg/l	mg/l	per hour	per hour
16.5	890	299	2024	1.13	3.38
31.4	900	280	1934	1.03	3.45
56.0	905	152	865	0.48	2.85

As would be expected, the basic composition of the sludge solids varied considerably between units. The basic composition represents the composition of the solids 23 hours after feeding. Figure 27 shows the dry weight percentages of carbohydrate, protein and PHB as well as the unaccounted for "other" material. The basic carbohydrate content of the sludge solids varied from 9.8 per cent at COD/N 16.5 to 36.9 per cent in the unit operated at COD/N 56.0. The
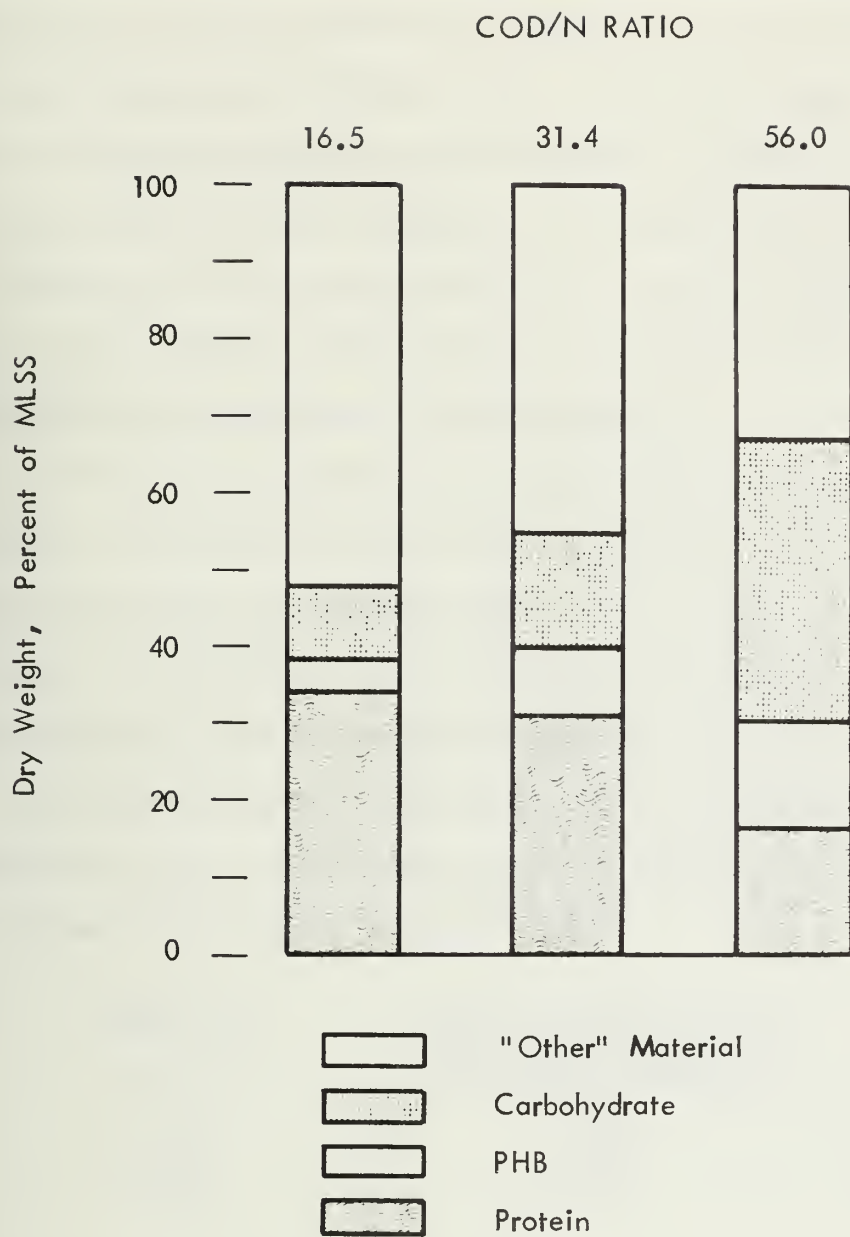
COD/N RATIO

FIGURE 27 BASIC COMPOSITION OF ACTIVATED SLUDGE ACCLIMATED TO
DIFFERENT COD/N RATIOS

PHB content, although making up a smaller percentage of the solids, increased with increasing COD/N ratio, reaching a maximum of 13.2 per cent of the solids in the COD/N 56.0 unit. Since these values represent a residual composition after 23 hours of aeration, it is interesting to note the high percentage of PHB remaining in the cell under a condition of such nitrogen deficiency as found in the COD/N 56.0 unit. The possiblity exists that some of the PHB is non-degradable under the conditions of the experiment.

There was also present in the sludge solids, material that was not mearured by any of the three tests for cell constituents. On a dry weight basis, this material makes up 52.3 per cent of the sludge solids in the COD/N 16.5 unit whereas in the nitrogen deficient COD/N 56.0 unit it constituted only 33.1 per cent of the solids. The oxidation level of this material, as calculated by its solids-COD/solids ratio, was found to be quite similar in all three units. The calculated values are shown below:

COD/N Ratio	Solids-COD/solids Ratio of "other" Material
16.5	1.09
31.4	1.10
56.0	1.10

The solids-COD/solids ratio of 1.10 for the "other" material would suggest a material that is more oxidized than protein, such as serum albumin (1.43), but more reduced than a carbohydrate, such as glucose (1.04). The exact nature of the other material was not

investigated but it will be discussed in somewhat more detail in a later section.

The variation in carbohydrates for each experiment is shown in Figure 28. The carbohydrate content was calculated on a weight of carbohydrate per weight of protein basis, similar to the method of calculation in experiments in the previous section. In the COD/N 16.5 unit, the carbohydrate content of the cell increased rapidly within 1 hour after the substrate was added, to a maximum of 1.03 gm/gm protein. During the next 5 hours, the carbohydrate content of the cell was reduced to nearly the same amount as was initially present. The unit that was operated at COD/N 31.4 contained cells that also increased in carbohydrate content. The maximum amount, 1.54 gm/gm protein, was reached within 2 hours after contact with the substrate. These cells, however, required a longer period of time to return to their original carbohydrate level. Both of these units approximately tripled the initial carbohydrate content before degradation occurred.

The nitrogen deficient unit, COD/N 56.0, exhibited a 4-hour lag period before synthesis of any reserve carbohydrates took place. According to Figure 26, the actual carbohydrate concentration was increasing during this period; however, the protein was also increasing at the same rate. As a result, there were no excess carbohyrates accumulated. The carbohydrates that were synthesized were probably structural carbohydrates. After the 4th hour, the
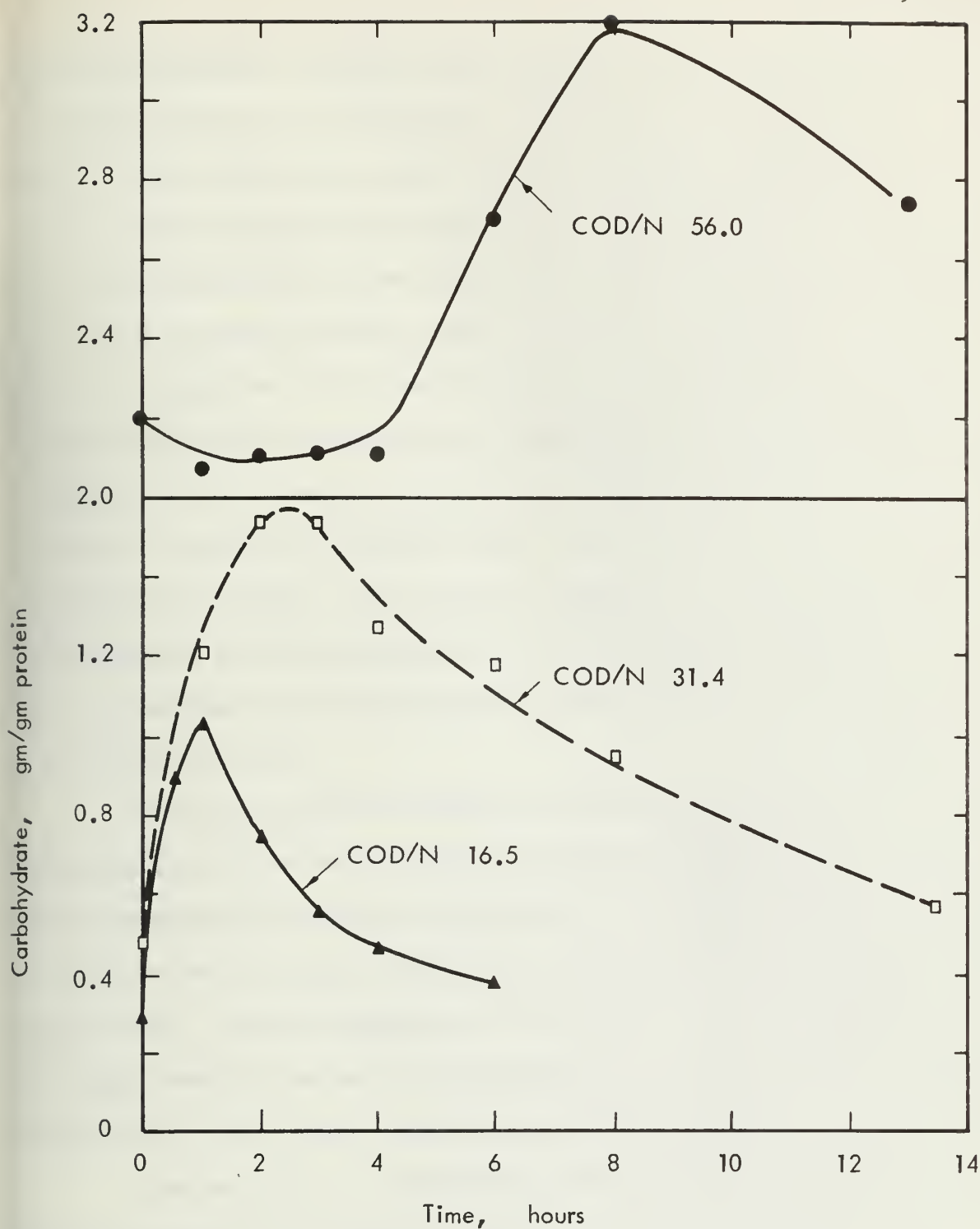
FIGURE 28   VARIATION IN CELLULAR CARBOHYDRATE DURING METABOLISM
OF SUBSTRATE BY ACTIVATED SLUDGE ACCLIMATED TO
DIFFERENT COD/N RATIOS

reserve carbohydrates increased from 2.2 gm/gm protein to a maximum of 3.2 gm/gm protein at the 8th hour. The initial carbohydrate content of this unit was more than 4 times the initial carbohydrate content of the two lower COD/N units. This increased initial carbohydrate content could have been attributed to the synthesis of extracellular capsular material without subsequent degradation. The possible presence of this extracellular material would not affect the ability of the organism to accumulate reserve carbohydrates but merely affect the rate of accumulation. The total quantity accumulated between the fourth and eighth hour was 1.01 gm carbohydrate/gm protein. This value was comparable to the 1.06 gm carbohydrate/gm protein accumulated by the unit operated at COD/N 31.4, and somewhat higher than the 0.74 gm carbohydrate/gm protein by the COD/N 16.5 unit.

The major difference between the units was the rate of carbohydrate synthesis and the rate of carbohydrate degradation. These parameters were inversely proportional to the COD/N ratio of the units and would greatly influence the operation of an activated sludge system. Systems operating at a low COD/N ratio would exhibit more rapid synthesis and more rapid degradation of carbohydrate storage products than units operating at high COD/N ratios.

The variation in intracellular PHB accumulation during substrate removal by the three activated sludge units is shown in Figure 29. The two units operated at lower COD/N ratios were quite similar. They rapidly accumulated the lipid polymer within the first
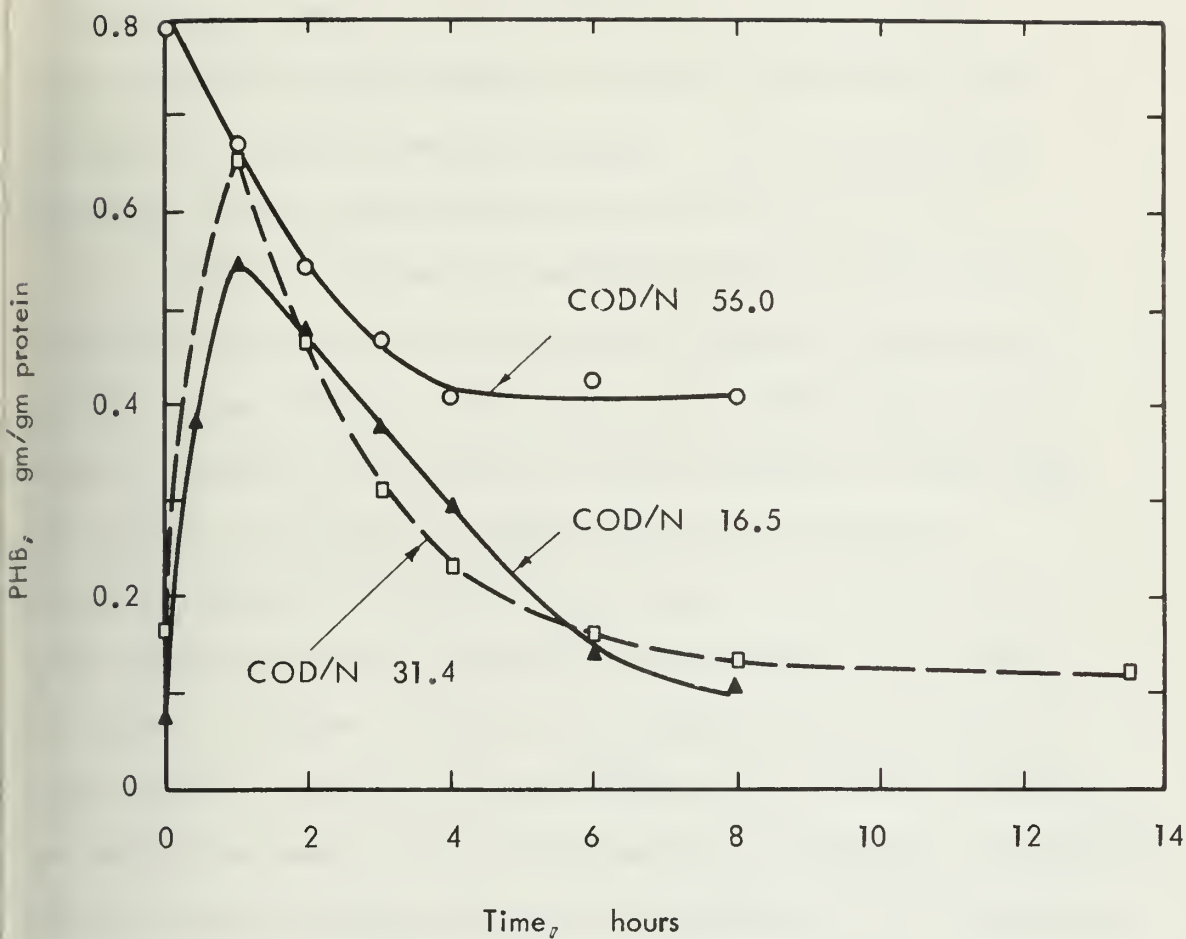
FIGURE 29 VARIATION IN CELLULAR PHB DURING METABOLISM OF
SUBSTRATE BY ACTIVATED SLUDGE ACCLIMATED TO
DIFFERENT COD/N RATIOS

hour and during the next 5 hours degraded the material back to the initial level. The complete cycle of synthesis and degradation required approximately 6 hours. Although some PHB was synthesized in the nitrogen deficient unit (COD/N 56.0), as shown by the slight increase in concentration in Figure 26, the weight of PHB per weight of protein actually decreased. This indicated there was no PHB storage under conditions of nitrogen deficiency.

The data obtained from each experiment were analyzed to find the per cent of substrate that was converted to carbohydrate and PHB storage products. As in the previous study which dealt with the influence of F/M ratio on storage products, a balance was made between the decrease in soluble COD and the increase in carbohydrate-COD plus PHB-COD. The increase in the COD of the latter two components was reported as a percentage of the soluble COD removed. In order to negate the influence of one constituent being used as a substrate for the synthesis of a second constituent, the balance was made at the point of maximum carbohydrate concentration. Table 11 shows the maximum conversion of soluble COD to carbohydrate-COD and PHB-COD. It can be seen that the COD/N ratio does not influence the per cent of soluble COD converted to carbohydrates. Each system converted approximately 26 per cent of the substrate into carbohydrates. However, the amount of substrate converted to PHB was considerably reduced under conditions of high nitrogen deficiency. This was probably due to the inability of the cells to synthesis enzymes associated with the synthesis of the lipid polymer.

TABLE 11

MAXIMUM CONVERSION OF SOLUBLE SUBSTRATE TO
CARBOHYDRATES AND PHB FOR SYSTEMS ACCLIMATED
TO DIFFERENT COD/N RATIOS

COD/N	Per Cent of Substrate Converted to	
	Carbohydrate	PHB
16.5	27.3	21.6
31.4	27.4	24.1
56.0	26.2	4.0

In summary, it can be said that the COD/N ratio influences the
storage and substrate removal capacity of activated sludge systems.
It appears that as the COD/N ratio increases from 16.5 to 31.4, the
activated sludge has a slightly greater capacity for storage. If the
total weight of storage material is added together, that is, the
carbohydrate plus the PHB, the unit operating at a COD/N ratio of 16.5
had a total storage of 1.22 gm/gm protein while the unit operating at
a COD/N ratio of 31.4 stored a total of 1.54 gm/gm protein. How much
greater this total storage could have been if the COD/N ratio were
increased in smaller increments between 31.4 and 56.0 is not known, but
it is known that at some point the storage of PHB is inhibited because
there was no PHB stored in the unit operated at a COD/N ratio of 56.0.
From these results, although they are somewhat limited in range, it
appears that the COD/N ratio does not influence the amount of cellular
storage as much as the F/M ratio. In the latter studies, the quantity
of storage products were, at the very least, doubled when the F/M ratio

was adjusted. In the present COD/N studies, however, no doubling in the quantity of storage products was observed.

Even though there was a deficiency of nitrogen in the unit having a COD/N ratio of 56.0, the substrate removal rate, on a per gram of protein basis, was not appreciably different when compared with the substrate removal rate of the nutritionally balanced systems. Provided that the settling ability of the sludge is not adversely affected, this would indicate that perhaps activated sludge systems can operate at much higher BOD/N ratios than have been previously considered. In the past, little attention has been given to the possibility that organic nitrogen may be recycled during the sludge reaeration process. When the mixed liquor is subjected to long periods of stabilization, autodigestion and cell lysis takes place thus making available organic nitrogen which may be used for subsequent periods of growth. Under a condition of high COD/N ratio, however, only the synthesis of essential nitrogen components, such as respiratory enzymes, will take place, and those which are considered as non-essential, like the enzymes involved in the storage of substrate, would not be synthesized. Nevertheless, this source of organic nitrogen should not be overlooked as it may be a contributing factor in operating units at high COD/N ratios. Washington and Symons (39) have indicated that the recycling of cellular nitrogen should be considered in establishing the optimum BOD/N ratio for the activated sludge process.

In the past there have been studies (38,39,51,52,53) in which the carbohydrate content of activated sludge has been reported. From

the results of the present study it may be implied that there are at least two identifiable carbohydrate fractions: an intracellular fraction, characterized by its relatively rapid synthesis and degradation, and a second fraction that is characterized by its slow build-up and non-degradability. The latter fraction may be considered as polysaccharide capsular material and is generally only observed in significant proportions when an activated sludge is acclimated to a nitrogen deficiency and/or under limited wasting conditions. Nevertheless, it should be kept in mind that there are at least two carbohydrate fractions associated with the cell and that the true storage carbohydrates are those that are rapidly accumulated and degraded.

3. Substrate

In any survey of literature pertaining to biological waste treatment, one almost always finds reference to the influence of the nature of the waste on the treatment process. It was, therefore, considered a major area of interest to determine the influence of substrate on the synthesis of storage products.

Three 2 liter fill and draw laboratory activated sludge units were seeded with mixed liquor from the stock glucose-yeast extract unit and operated on a 50 per cent wasting schedule. One unit was acclimated to glucose, the second to acetic acid, and the third unit to glutamic acid. The substrate concentration for all units was 2000 mg/l as COD. Inorganic nitrogen was supplied to all units in a concentration of 500 mg/l. The COD/N ratio for the glucose and the acetic acid units

was 18.0 and for the glutamic acid unit it was 6.5. The F/M ratio of the 3 units was 2.9.

All units were acclimated for a period of more than 10 days. The modified sludge volume index for three days prior to the experiment averaged 60, 69 and 36 for the glucose, acetic acid, and glutamic acid units, respectively. The pH of the three units during the three days prior to the experiment averaged 7.0, 8.4, and 8.5, respectively. The experimental procedure and sampling protocol is described in Chapter V.

The response of the systems during metabolism of the three substrates is shown in Figures 30, 31, and 32. Analyses were performed for MLSS, soluble COD, carbohydrates, protein, and PHB.

There was no PHB in any of the systems investigated, despite the fact that each had been seeded with mixed liquor that was capable of synthesizing PHB. It was apparent that the organisms responsible for PHB synthesis were not a part of the biota in the units under study. This has been discussed previously when infrared spectrums were obtained on these same sludges.

Although the carbohydrate content of the cells in all three units showed an increase, the glucose unit was the only one to exhibit a substantial increase. The data were replotted on the basis of gm carbohydrate/gm protein and these results are shown in Figure 33. The only unit that showed any carbohydrate storage function was the glucose unit. In a 3-hour period, the amount of carbohydrates increased from 1.11 gm/gm protein to 2.95 gm/gm protein. During the next 5 hours, 65.5 per cent of the accumulated carbohydrates were degraded.

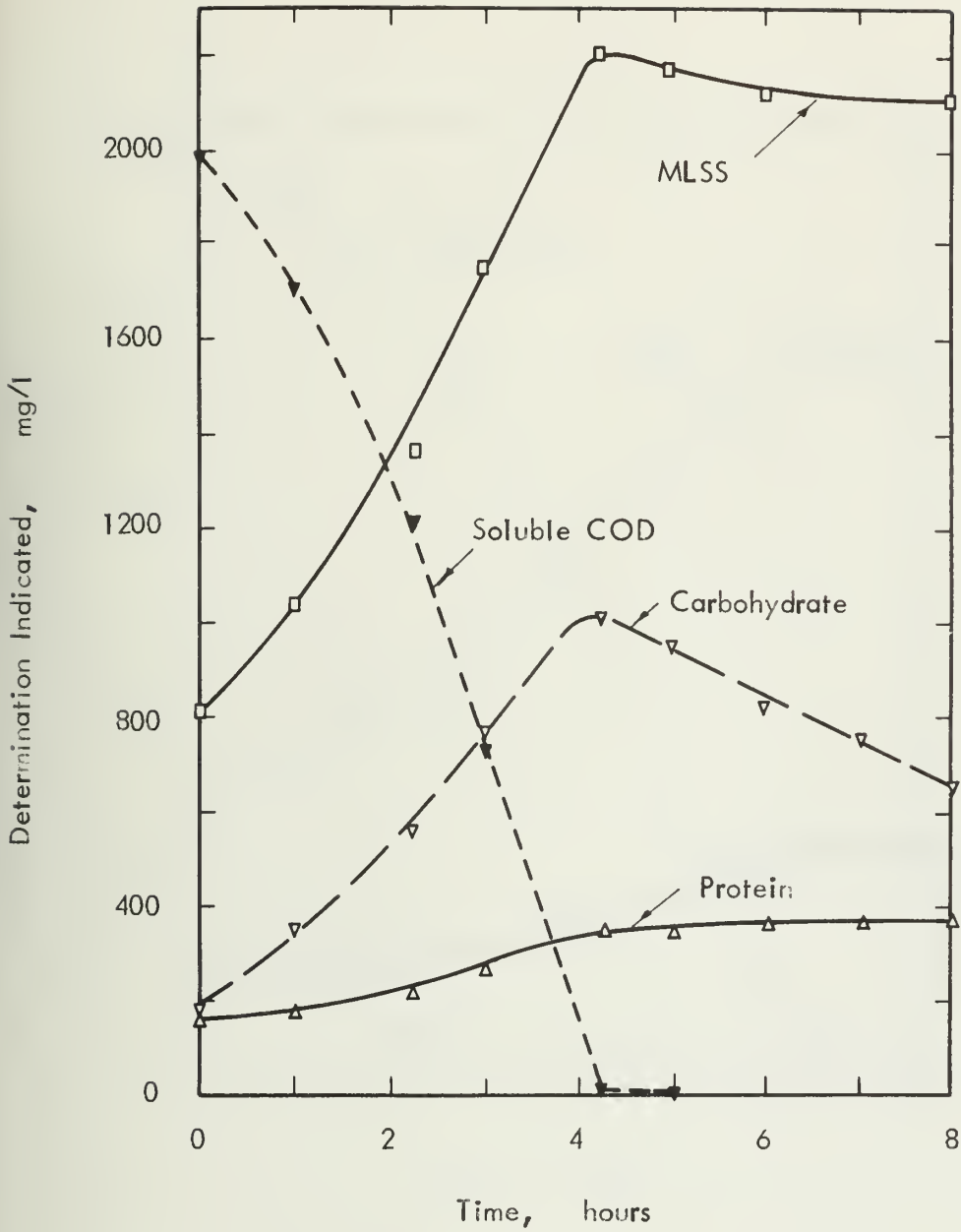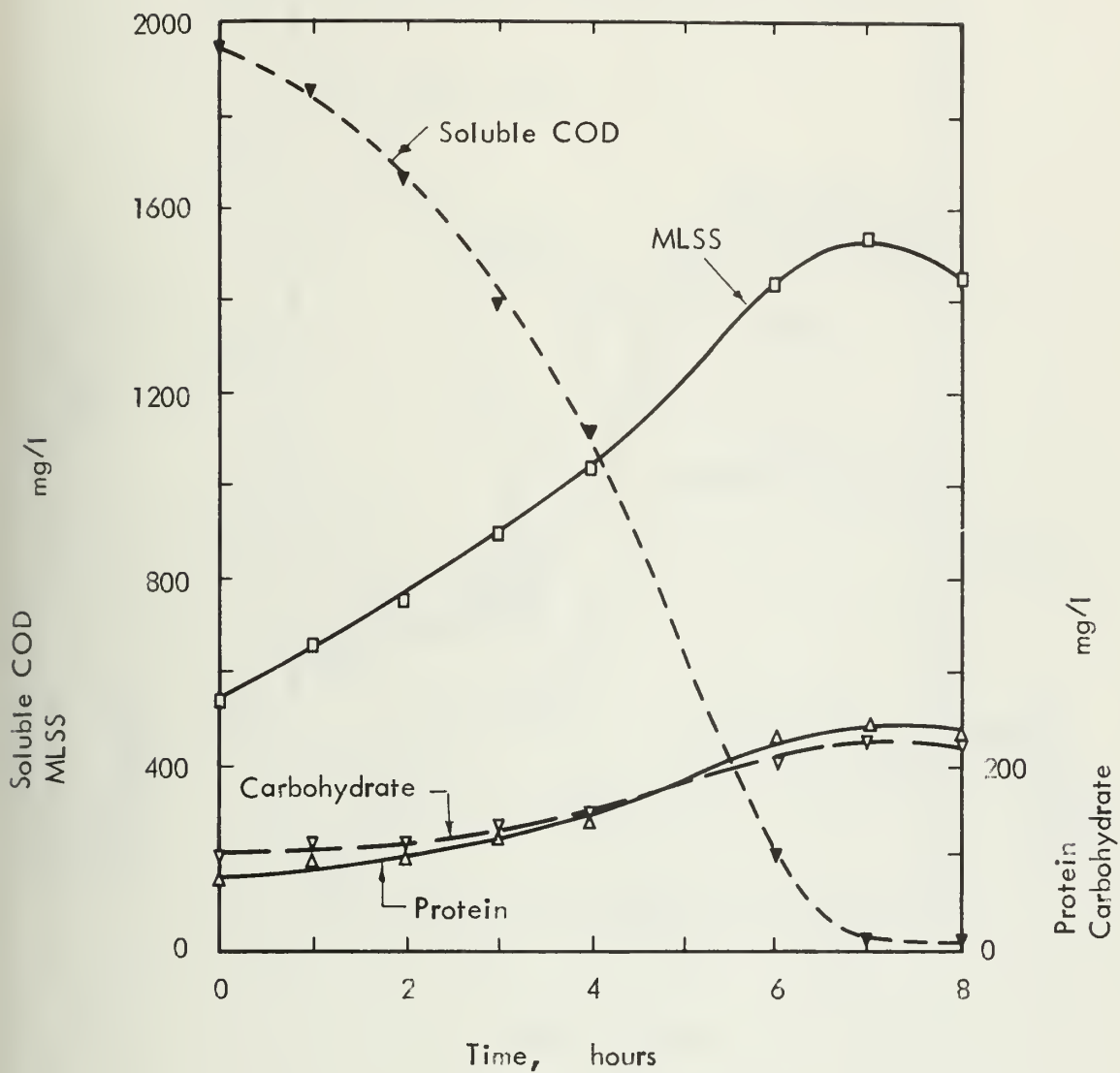FIGURE 30   SYSTEM RESPONSE DURING METABOLISM OF GLUCOSE BY AN ACCLIMATED ACTIVATED SLUDGE

FIGURE 31   SYSTEM RESPONSE DURING METABOLISM OF ACETIC ACID
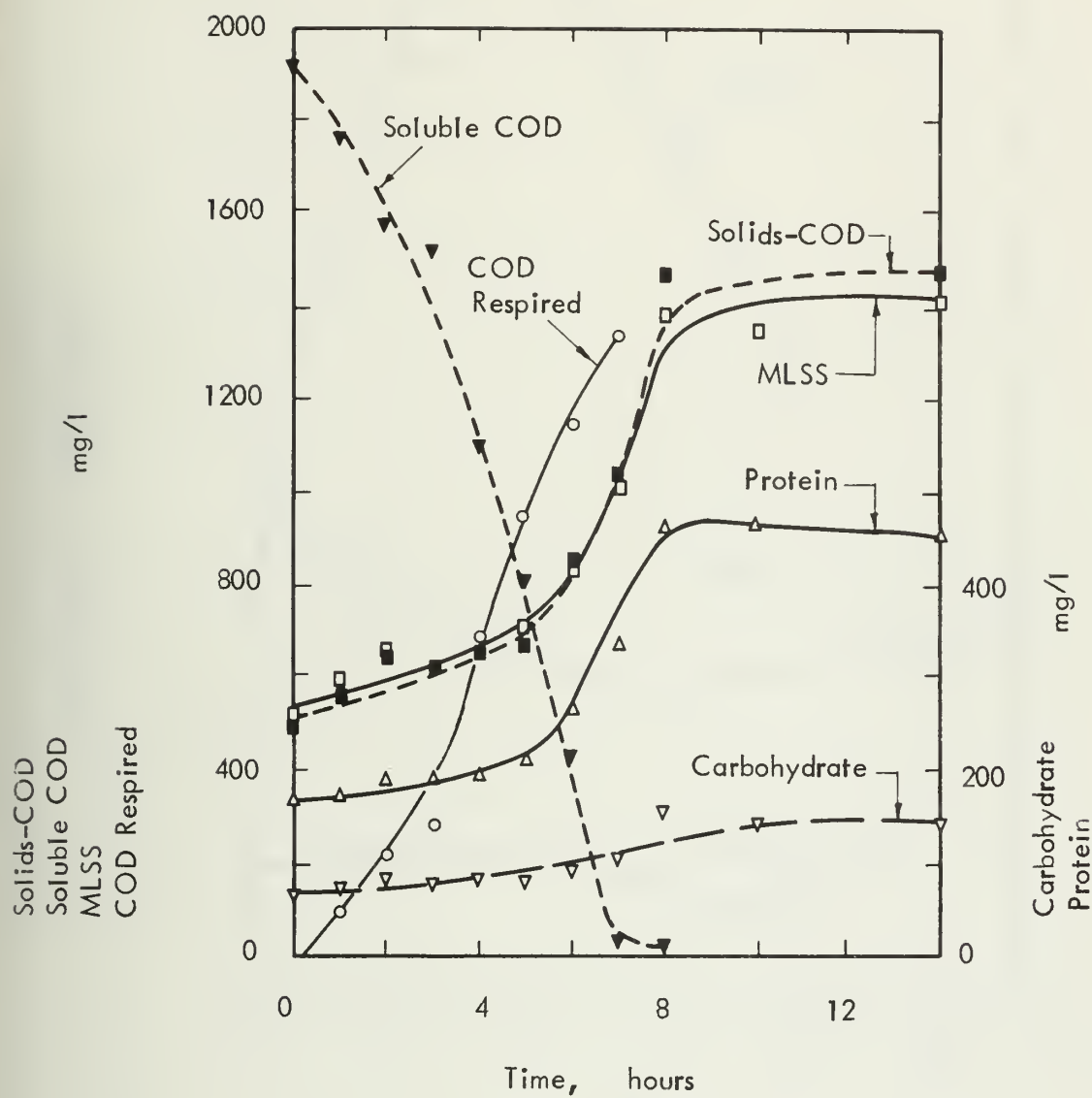BY AN ACCLIMATED ACTIVATED SLUDGE

FIGURE 32   SYSTEM RESPONSE DURING METABOLISM OF GLUTAMIC ACID
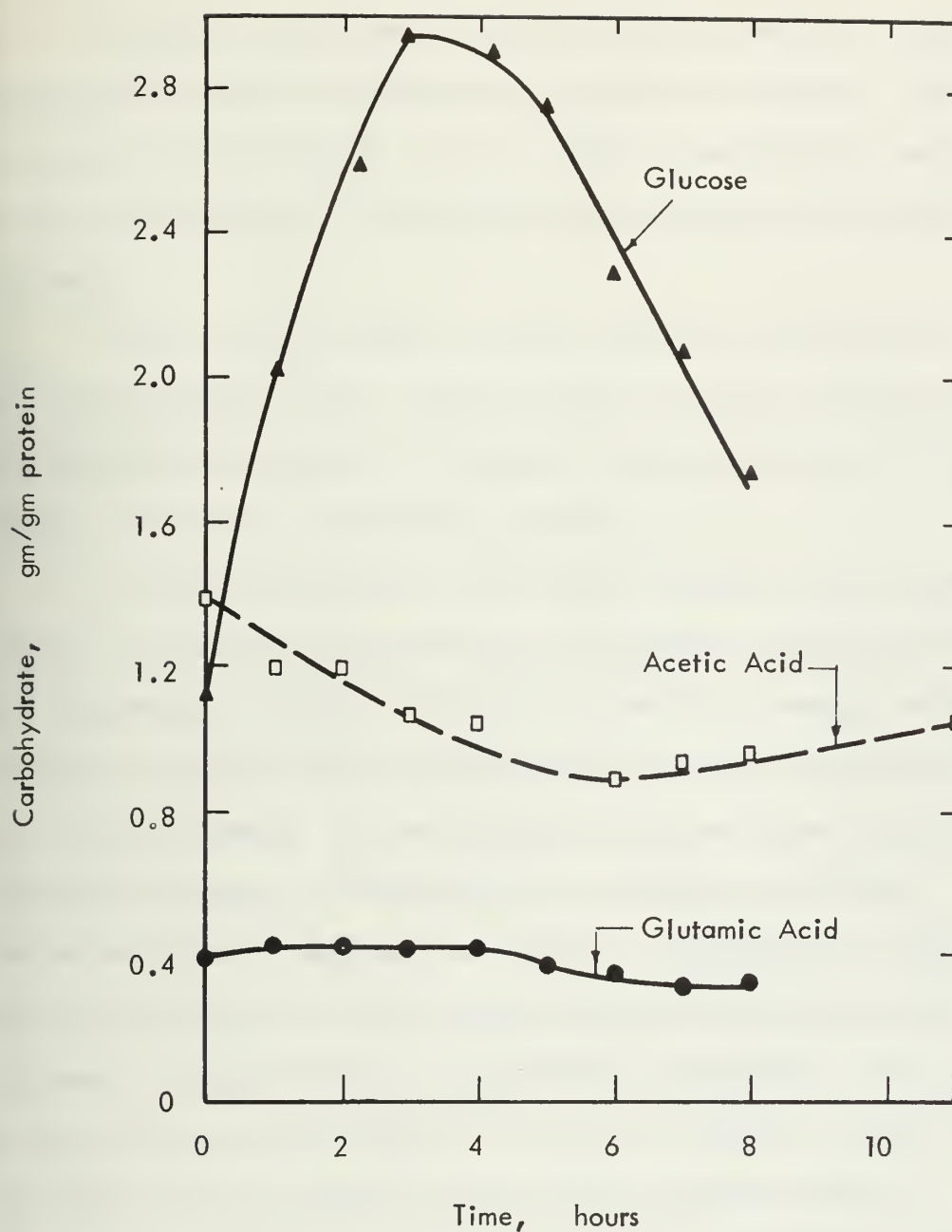BY AN ACCLIMATED ACTIVATED SLUDGE

FIGURE 33   VARIATION IN CELLULAR CARBOHYDRATE DURING METABOLISM
BY ACTIVATED SLUDGES ACCLIMATED TO DIFFERENT SUBSTRATES

Microbial cells from the acetic acid unit actually showed a decrease in the weight of carbohydrates per weight of protein. The glutamic acid unit, similarly, did not exhibit a carbohydrate storage function since the weight of carbohydrates per weight of protein did not increase.

Cells in the glutamic acid unit respired a considerable amount of the substrate COD. During the first 7 hours, 71.5 per cent of the substrate was respired. Thereafter, the major portion of the substrate removed went into protein synthesis.

The basic composition of the cells from each unit is shown in Figure 34 along with the composition of the cells when all the substrate was removed. The data indicate that the major storage component of the cells from each sludge was related to the type of substrate to which it was acclimated. The carbohydrate-fed mixed liquor contained the highest percentage of carbohydrate and the amino acid sludge contained the highest percentage of protein. A comparison of cells after all the substrate had been removed showed that the acetic acid and glutamic acid units did not change much in composition. This would indicate there was little storage of any kind in either of these systems. On the other hand, the glucose sludge showed a definite change in composition. This change was attributed to the accumulation of carbohydrate storage products. Three hours from time zero the composition of the cells was so close to that of the 8-hour composition that it was not included in Figure 34.
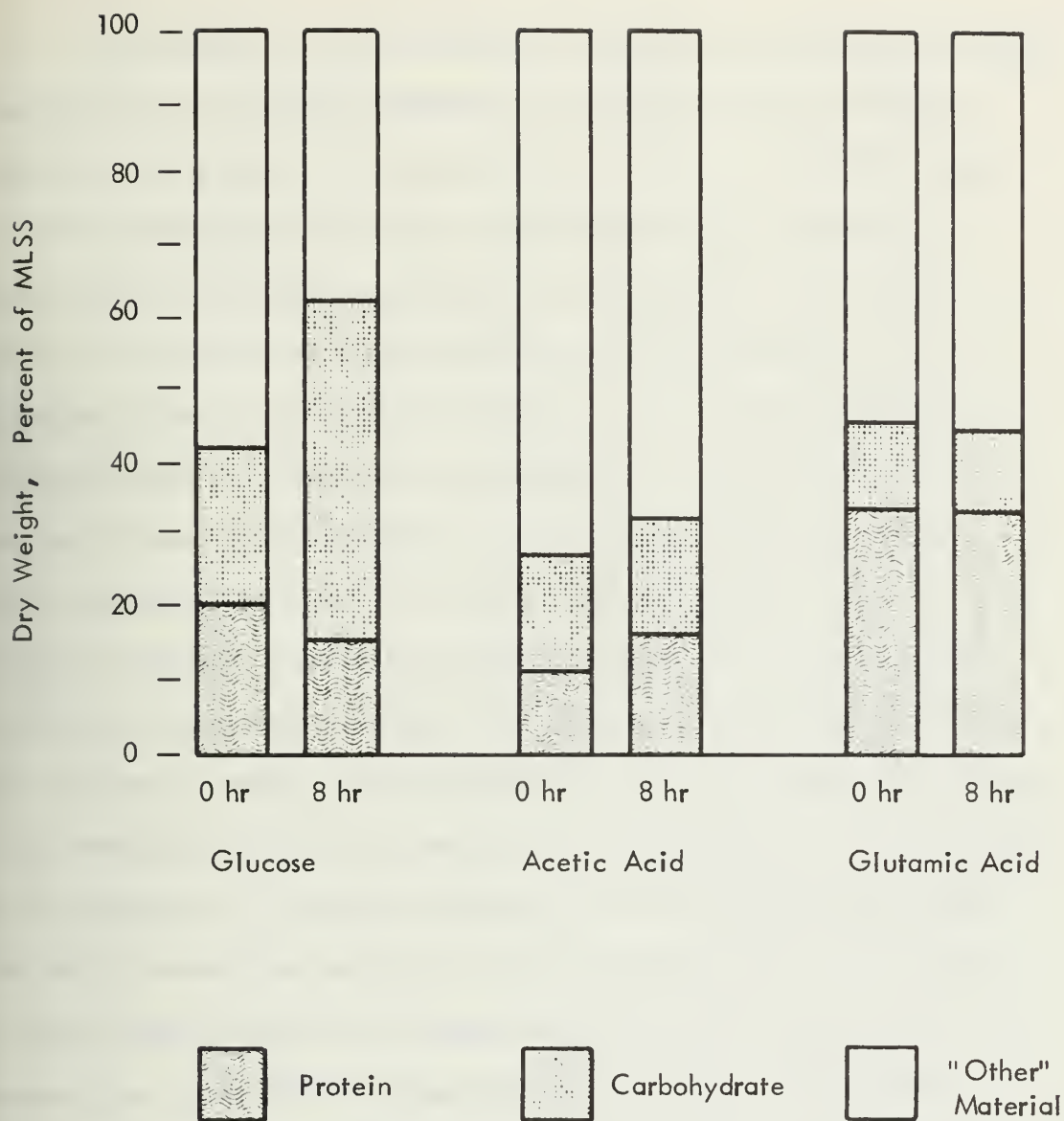
FIGURE 34 SOLIDS COMPOSITION BEFORE FEEDING AND 8 HOURS AFTER FEEDING OF ACTIVATED SLUDGE ACCLIMATED TO DIFFERENT SUBSTRATES

In summary it can be said that the data have provided evidence indicating that the storage phenomena in activated sludge systems is dependent on the nature of the waste being treated. In systems where the wastes are mainly amino acid or lower fatty acid in nature, no carbohydrate or PHB storage material were accumulated. This would possibly be true also for higher molecular weight substances of the same classes, that is, proteins and lipids, if they were hydrolyzed to their lower amino and volatile acid components. In a system where the wastes are primarily carbohydrate in nature with a limited organic nitrogen content, there would be no PHB storage but there would be carbohydrate storage. For wastes that are balanced in carbohydrates and protein, such as was available in the stock unit or that could be found in domestic sewage, one could expect to find a considerable amount of both carbohydrate and PHB material to be accumulated. It is believed that the phenomena of substrate storage in an activated sludge system is intimately associated with the type of microorganisms in the system. It is particularly evident in systems that are able to store PHB. The organisms that store PHB are dependent on the presence of preformed amino acids and/or peptides for growth. Without this form of organic nitrogen, the organisms responsible for the synthesis of this reserve material are washed out of the system and thus the activated sludge loses its ability to store PHB.

It appears from the information presented that the contact stabilization process, or any other process employing reaeration, may not be the most efficient means for treating wastes that have a high

protein content. There would be no reason for the reaeration step since the major portion of the substrate is respired rather than stored.

C. Influence of Storage Products on System Operation

1. Substrate Removal

At the cessation of a period of active assimilation, when the exogenous food supply is exhausted, activated sludge organisms begin to degrade some of the newly synthesized material. This was demonstrated, to some extent, in previous sections. The results of this degradation may be the release of carbon dioxide or the synthesis of any protoplasmic constituent including enzymes. This phase of the activated sludge waste treatment process is generally referred to as the stabilization period. It may take place in the same aeration tank where the substrate had been removed (conventional activated sludge process), in which case, the mixed liquor solids are settled, removed from the waste stream, and then reaerated.

In order to optimize the activated sludge process, it is important to know which organic fractions of the cells are being degraded and to relate this degradation to the ability of the organisms to remove substrate. Accordingly, an experiment was designed to measure the change in PHB, carbohydrates, protein, mixed liquor solids and solids-COD during the period of stabilization. The substrate removal capacity of the endogenously aerated sludge was determined by removing mixed liquor samples periodically during the stabilization period and contacting them with fresh substrate for 20 minutes. At the end of this time, the

reduction in soluble COD was measured as the "contact COD removed."
The experimental procedure and sampling protocol are described in
Chapter V.

The results are shown in Figure 35. It can be seen that
the organisms exhibited an increasing ability to remove substrate as
the period of stabilization increased. At the beginning of the
stabilization period, the organisms were able to remove 17.7 per cent
of the substrate COD upon exposure to fresh feed for a 20-minute
contact period. After 11 hours of stabilization, during which time
all of the storage carbohydrates and PHB were depleted, the organisms'
were able to remove 51.9 per cent of fresh substrate COD in a 20-minute
contact period. Additional stabilization did not result in any further
substrate removal ability.

During the 11-hour period of stabilization, there was an
almost constant rate of carbohydrate and PHB reduction. The carbohy-
drates were reduced from 586 mg/l to 179 mg/l and the PHB decreased
from 257 mg/l to 39 mg/l. When plotted as grams of carbohydrates and
PHB per gram of protein, Figure 36, the same relationship existed, i.e.,
a decrease in both of these storage materials during stabilization.
The increased capability for the removal of substrate (gm COD removed/
gm protein) also showed the same trend as in Figure 35.

The decrease in protein concentration was not in agreement
with previous studies already discussed, nor with the findings of other
investigators. Gaudy and Engelbrecht (9) and Siddiqi (95) found an
increase in protein when the carbohydrate concentration decreased. In
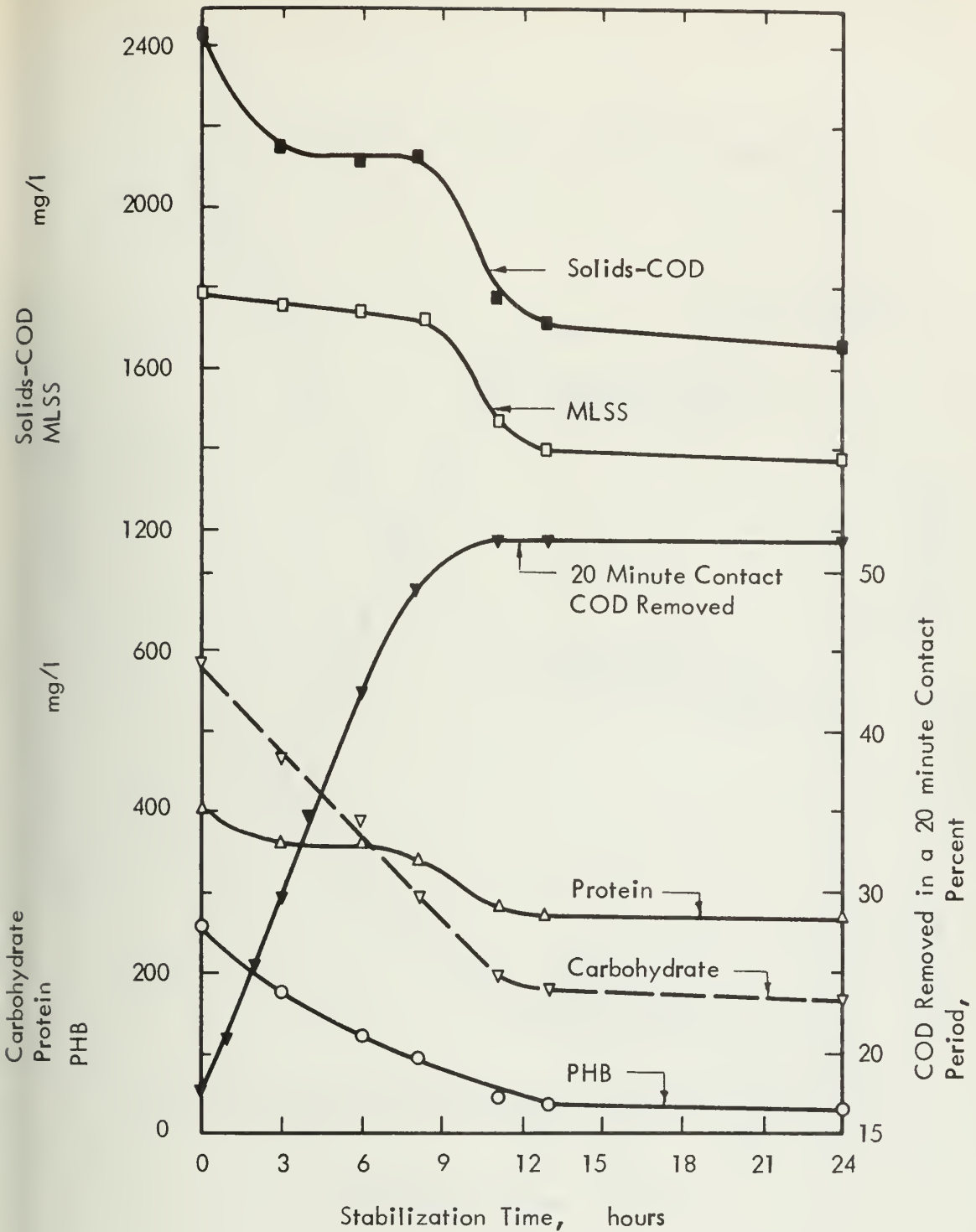
FIGURE 35   SYSTEM RESPONSE DURING ACTIVATED SLUDGE STABILIZATION
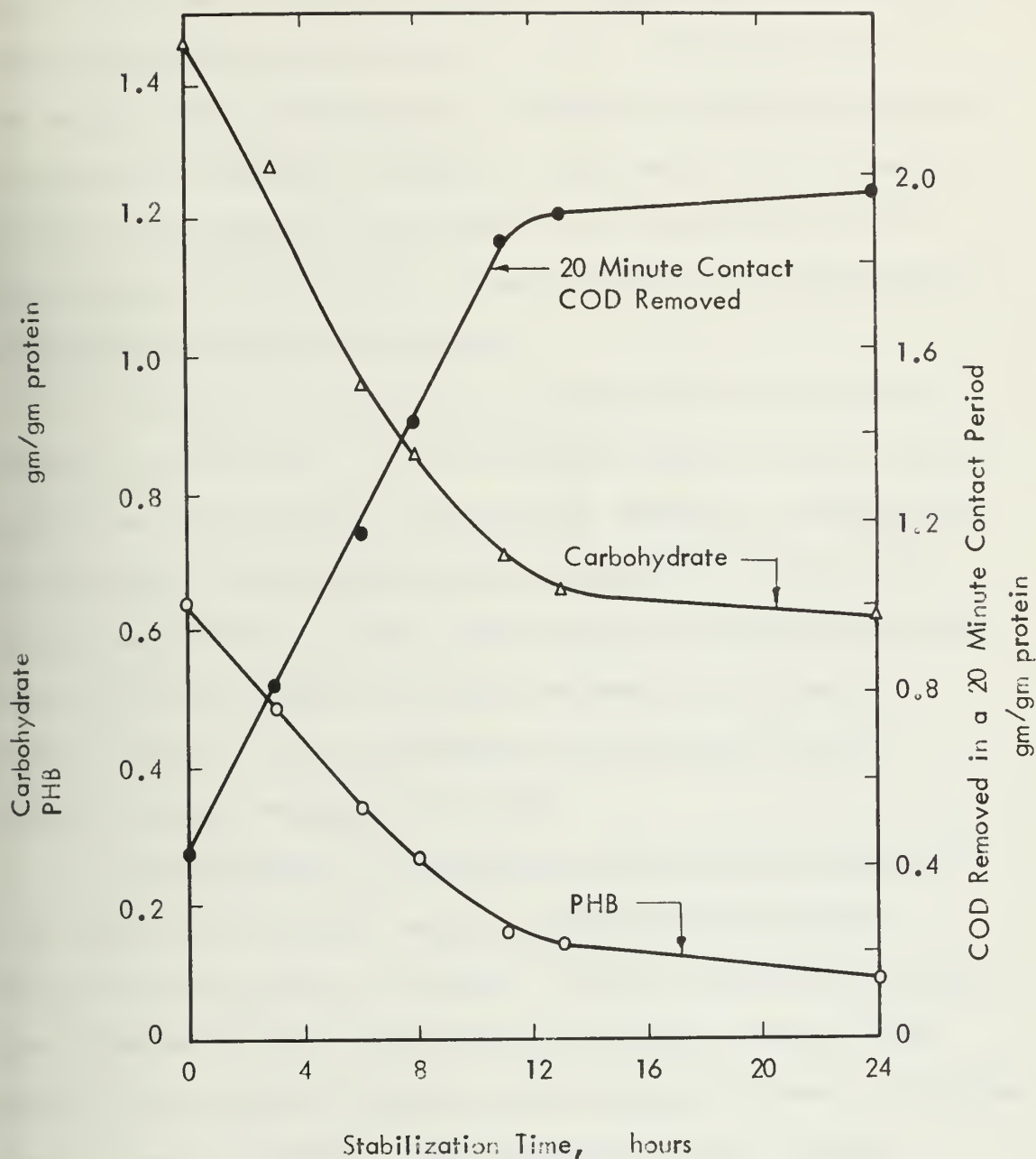IN A FILL AND DRAW UNIT

FIGURE 36 VARIATION IN CELLULAR CARBOHYDRATE, CELLULAR PHB AND
SUBSTRATE REMOVAL ABILITY DURING ACTIVATED SLUDGE
STABILIZATION IN A FILL AND DRAW UNIT

a preliminary experiment of the present work, designed to follow the changes in cellular constituents during the stabilization period, the protein concentration was observed to increase slightly during the first several hours of stabilization. Whether the change in protein is an increase or a decrease, the amount is quite small in either case, especially when compared to the change in the carbohydrate and PHB components of the sludge solids. It was, therefore, not considered of major interest in evaluating the data.

The solids concentration also decreased during the initial 11 hours of stabilization. It can be observed that the total decrease in solids was not equivalent to the combined decrease in carbohydrates, protein, and PHB. The decrease in carbohydrates was 388 mg/l; PHB, 212 mg/l; and protein, 123 mg/l. These cellular constituents decreased a total of 723 mg/l whereas the solids decreased only 324 mg/l. Obviously, 399 mg/l of the constituents were being used for the synthesis of other components of the cell.

If resynthesis of intracellular components was taking place in the cell, then the rate of carbon dioxide production might be expected to be low during this period. The high requirement for energy during resynthesis is generally supplied from carbon-energy storage products, such as PHB and glycogen, and not through the means of terminal oxidation (the Krebs Cycle). After resynthesis ceased, the CO_2 production would be at a maximum indicating that energy was being supplied from terminal oxidation. To test this theory, carbon dioxide measurements were made on activated sludge that had been contacted with

substrate for 1.25 hours according to the procedure previously outlined. As shown in Figure 37, the rate of carbon dioxide evolution increased during the first 10 hours of stabilization and then remained constant. These data, along with the data on solids balance, strongly implied the existence of a synthetic response by activated sludge organisms during the stabilization period.

No attempt was made to identify the material that was being synthesized during stabilization; however, several things can be said about it. Besides the fact that it is synthesized during sludge stabilization, the oxidation level of the material was 1.17, i.e., the solids-COD/solids ratio of a 24-hour sample. This value is within 6 per cent of the average values obtained in previous experiments. In the study on the effect of loading ratio on substrate storage, the solids-COD/solids ratio of the "other" material was 1.09, 1.10 and 1.1 for F/M ratios of 0.78, 1.52, and 7.25, respectively. In the COD/N study, all units, regardless of the COD/N ratio, contained "other" material that had a solids-COD/solids ratio of 1.10. The close agreement between the values for all these experiments indicates that environmental control has little to do with the nature of the "other" material. Furthermore, whether the "other" material constituted 30 or 50 per cent of the cell, it has the same solids-COD/solids ratio.

The results of these studies indicate that the stabilization period in the activated sludge process is a dynamic state of metabolism in which degradation as well as synthesis of cellular materials takes place.
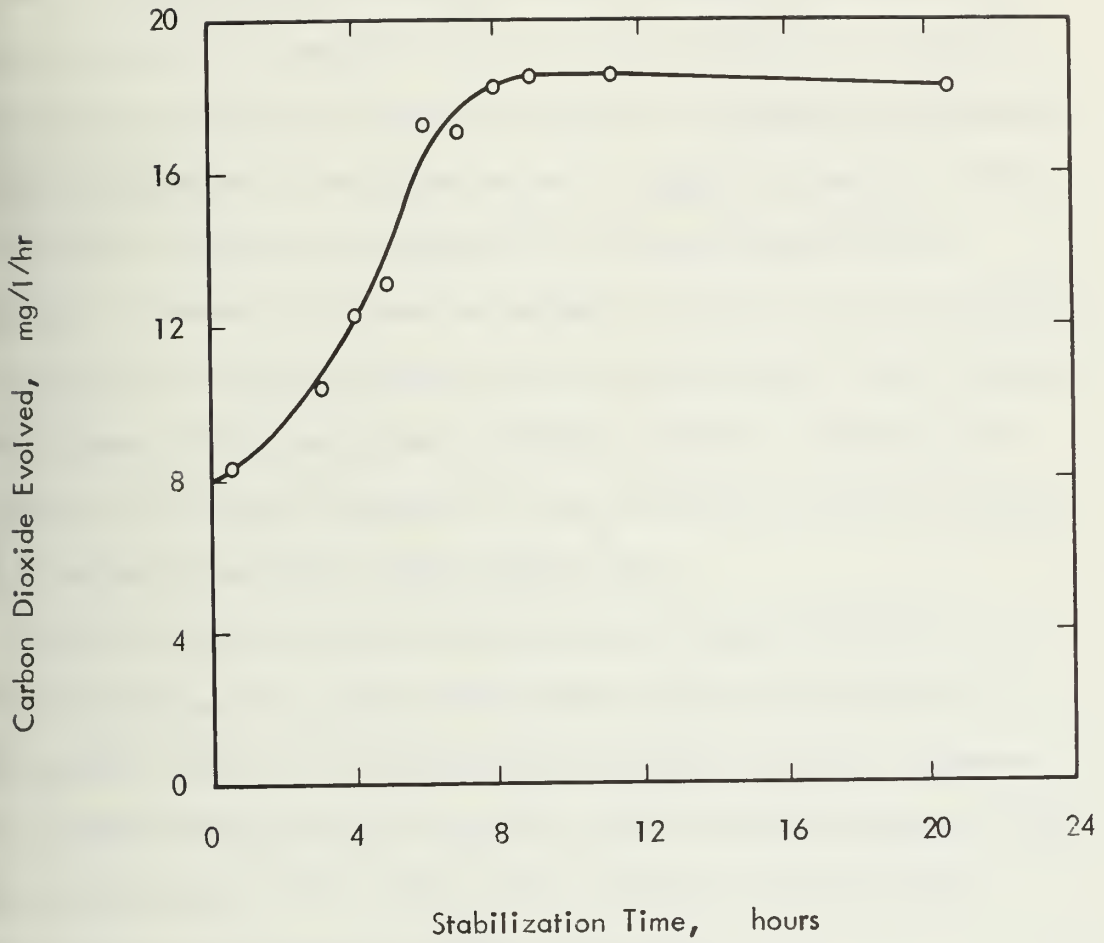
FIGURE 37   RATE OF CARBON DIOXIDE PRODUCTION DURING SLUDGE
STABILIZATION IN A FILL AND DRAW UNIT

The period of endogenous metabolism is an area of investigation which has been given little attention by sanitary engineers. This is due, in part, to an innate comparsion that is usually make between endogenous metabolism and substrate removal. Whereas the period of substrate removal is associated with the rapid synthesis of cellular constituents in the mixed liquor, the period of endogenous respiration is generally assumed to be characterized by slow autodigestion of the solids and is, therefore, considered as a rather placid state of metabolism. However, this does not appear to be true. It is recognized that, in pure cultures, synthesis can be an active part of the stationary phase of growth (97), particularly enzyme synthesis. Recently, the synthesis of respiratory enzymes has been indicated during the stabilization period in activated sludge systems (95).

The data presented here indicate that an unknown material is synthesized during the stabilization period and it is not protein, carbohydrate, or PHB. The material has an oxidation level, expressed as its solids-COD/solids ratio, which is lower than the oxidation level of the mixed liquor solids. This would indicate that the "other" material is an more oxidized portion of the cell.

The data also indicate that the ability of the organisms to remove substrate is related to the amount and type of cellular constituents within the cell. With increasing amounts of intracellular carbohydtates and PHB storage products, the cell is less able to remove significant amounts of substrate. In the design of activated sludge

systems, consideration should therefore be given to the period of
sludge stabilization so that the maximum amount of substrate removal
can be obtained when the reaerated sludge is returned to the aeration
tank. The optimum period of sludge stabilization may well be defined
as the amount of time mecessary to degrade intracellular storage
products to a minimum level. However, there are other considerations,
both economic and operational, that may influence the stabilization
period. One is the direct relationship between cost and detention time.
If the stabilization time is unreasonably high, the cost will follow
the same pattern. And in some instances the settling characteristics
of the sludge solids may be influenced by the stabilization time.

2. Sludge Settling Characteristics

In the operation of an activated sludge treatment plant it is
important to maintain a sludge that has good settling characteristics.
When organic matter enters the treatment plant it is transformed
primarily into mixed liquor solids. The only way that the objectionable
organic matter can then be removed is by separating the solids from the
liquid. To maintain process efficiency and to insure a high quality
effluent that is low in suspended solids, the mixed liquor is subjected
to a quiescent period in which the solids flocculate and settle. The
solids are then removed from the bottom of the tank and the clarified
waste stream is discharged from the top of the tank. Without good
settling characteristics, the solids may be lost in the effluent thus
resulting in a decreased efficiency of the activated sludge process.

An investigation was, therefore, conducted to determine if storage products, both carbohydrate and PHB, would effect the sludge settling characteristics of activated sludge.

The fill and draw type of operation did not readily lend itself to acquiring information pertaining to the settling characteristics of activated sludge systems. It was decided to use a continuous flow apparatus consisting of a separate contact aeration tank and a stabilization tank, as shown in Figure 3. Feed was metered to the 500-ml aeration tank at the rate of 140 ml/hr. The overflow from the contact aeration tank was further aerated in a stabilization tank. This unit had an adjustable overflow outlet by which the volume could be set at any level between 1 and 4 liters.

The flow rate through the contact aeration tank and the substrate concentration in the feed reservoir was set up in such a way as to provide a constant and uniform source of non-settling mixed liquor that was devoid of substrate. This mixed liquor was then subjected to varying periods of stabilization by adjusting the height of the over-flow outlet in the stabilization tank. At each level, 1, 2, 3, and 4 liters, the sludge settling characteristics and other parameters were measured. The levels corresponded to theoretical detention times of 7.1, 14.3, 21.4, and 28.6 hours of stabilization.

The contact aeration tank was seeded with activated sludge from the stock fill and draw unit. The concentration of organic substrates, mineral salts and buffer in the reservoir is shown in Table 6. The loading rate on the contact aeration tank was 12.6 gm COD/(gm MLSS)(day).

The experiment was begun by setting the overflow outlet in the stabilization unit at 4 liters and allowing the unit to come to equilibrium over a period of 10 days. After sampling the stabilization unit at this level, the overflow was dropped to the 3-liter level and the unit allowed to come to equilibrium for a period of time equivalent to 5 volume displacements. After again sampling, the overflow level was set at the 2-liter level and the equilibrium procedure repeated. The same procedure was used at the 1-liter level. At each level 3 samples were obtained, 1 volume displacement apart in time, and averaged. Each sample of mixed liquor was analyzed for MLSS, solids-COD, soluble COD, carbohydrates, PHB, and protein using the same procedure as shown in Figure 6. In addition, a 100-ml sample was pipetted from the stabilization unit into a graduate cylinder and the solids allowed to settle for 30 minutes. At the end of this time, the settled sludge volume was recorded and a 25-ml sample of the supernatant was obtained by placing the pipette tip at the 50-ml mark on the graduate cylinder. The solids content of the supernatant sample was determined in the same manner as for MLSS.

The results are shown in Figures 38 and 39. Samples for zero stabilization time were obtained directly from the contact aeration tank.

The mixed liquor suspended solids decreased linearly from a maximum of 2027 mg/l to 840 mg/l as the stabilization time increased from zero to 28.6 hours. The soluble COD increased slightly from 250 mg/l to 333 mg/l in 14.3 hours and then decreased to approximately
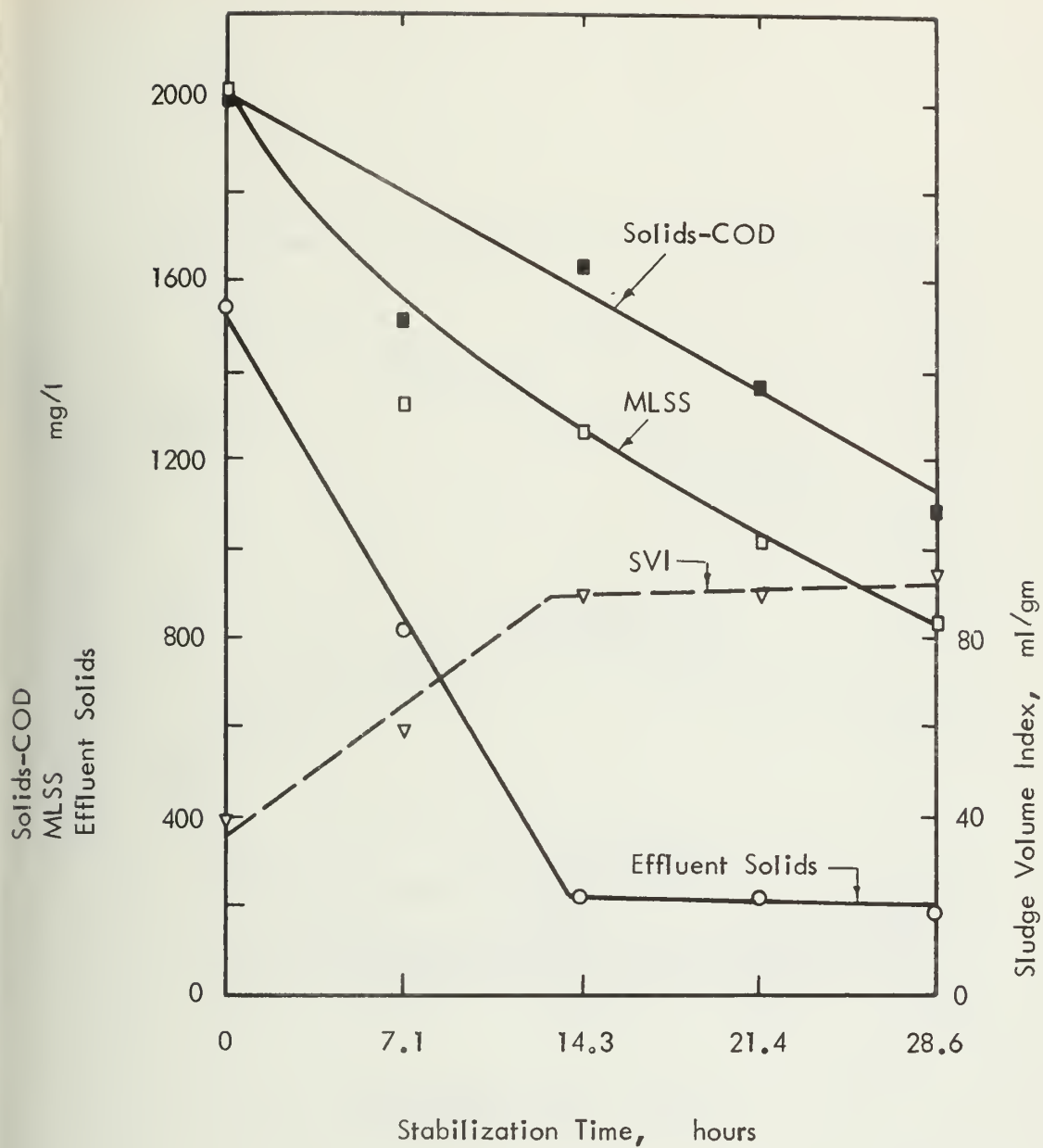
FIGURE 38   VARIATION IN THE SLUDGE VOLUME INDEX (SVI) AND SOLIDS
DURING ACTIVATED SLUDGE STABILIZATION IN A
CONTINUOUS FLOW UNIT
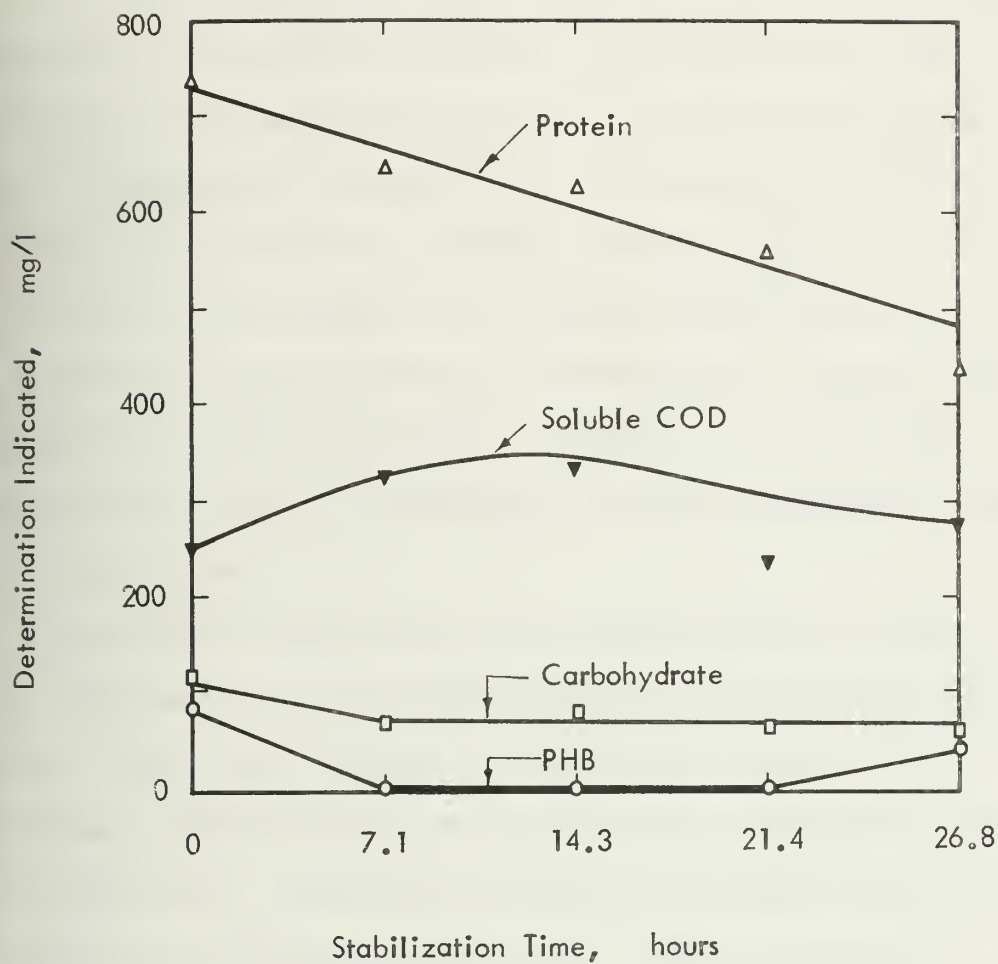
FIGURE 39   VARIATION IN CELLULAR CONSTITUENTS AND SOLUBLE COD
DURING ACTIVATED SLUDGE STABILIZATION IN A
CONTINUOUS FLOW UNIT

50 mg/l after 28.6 hours. Whatever changes took place in the cellular constituents of the mixed liquor during the stabilization period could not be attributed to the presence of an exogenous substrate supply because there was essentially no COD removed in the stabilization unit under any conditions. The feed reservoir COD was 3795 mg/l which was reduced to 250 mg/l in the contact aeration unit. This represents a residual COD of 6.6 per cent of the feed COD, which is consistent with published information (10).

The solids-COD decreased from a maximum of 2021 mg/l at time zero to 1082 mg/l after 28.6 hours of stabilization. Protein similarly decreased from 735 mg/l to 438 mg/l. Carbohydrate concentration decreased from 114 mg/l to 56 mg/l while the PHB concentration decreased from 82 mg/l to zero.

In the contact aeration tank, the effluent solids, i.e., the solids remaining in the supernatant after 30 minutes settling, were very high. There were 1542 mg/l effluent solids in the settled aeration tank effluent which represented 85.7 per cent of the total mixed liquor solids. The settling quality of the mixed liquor in the aeration tank was obviously very poor. After 14.3 hours of stabilization the effluent still contained 200 mg/l of solids which represented 17.8 per cent of the mixed liquor suspended solids. Additional stabilization did not decrease effluent solids concentration. From the data, it appeared that the optimum period of stabilization necessary to obtain good settling for this particular activated sludge would be 14.3 hours.

The sludge volume index was not a good measure of the clarifica-
tion property of the activated sludge since the supernatant liquor
contains such a wide variation in solids content. Under these
circumstances it is entirely possible to obtain the same SVI for a good
settling sludge with clear supernatant as for a dispersed sludge
with a highly turbid supernatant. For example: given two entirely
different activated sludge units, both containing 2000 mg/l MLSS, in
which 50 per cent of the solids settle to 100-ml volume (in a 1000-ml
mixed liquor sample) in one unit and 100 per cent of the solids settle
to 100-ml volume in the second unit. Both of these units have an SVI
of 50; however, the supernatant from the former unit contains 1000 mg/l
of solids whereas in the latter unit the supernatant is free of solids.

Obviously the operation of the latter unit is to be preferred but
the measurement of SVI would not provide the means for identifying
which of the two units had the better effluent. To overcome this diffi-
culty in clarification index (CI) was introduced which optimizes the
conditions for good clarification.

The optimum condition for clarification is described when there
is a minimum of solids in the supernatant and the settled sludge solids
are contained in a minimum volume. The CI was, therefore, calculated
as the product of these two, i.e., the per cent of total MLSS in the
supernatant multiplied by the volume of sludge solids that settle in a
100-ml sample of mixed liquor. For example, the aeration tank mixed
liquor solids to 7.7 ml and the supernatant contained 85.7 per cent of

the MLSS after 30 minutes settling. The CI was, therefore, 7.7 x 85.7 = 660. The lower the CI the better the clarification properties of the activated sludge. For the purpose of this study, the clarification index provided a good measure of the flocculation and clarification property of the sludge, however, the clarification index is neither applicable to all conditions of sludge flocculation nor can it be used to absolutely describe the settling properties of the sludge.

The variation in the CI is shown in Figure 40 along with the cellular constituents expressed as a per cent of the MLSS. The CI has the same trend as the effluent solids concentration (Figure 39) and also indicated that a stabilization period of 14.3 hours was needed to provide optimum sludge settling. As the stabilization period increased, the protein content of the MLSS increased from 36.2 per cent to 55.0 per cent even though the total quantity of protein decreased from 730 to 430 mg/l. This suggested that reserve materials were being degraded at a more rapid rate than protein. However, from the variation in carbohydrate and PHB content of the solids, it appeared that neither of these materials could be implicated as the reserve material. The so-called "other" material, i.e., the material that was neither carbohydrate, protein nor PHB, was obviously decreasing as the protein was increasing. It was possible that this "other" material influenced the settling characteristics of the sludge; however, there was no way of identifying the "other" material and confirming its influence on settling.
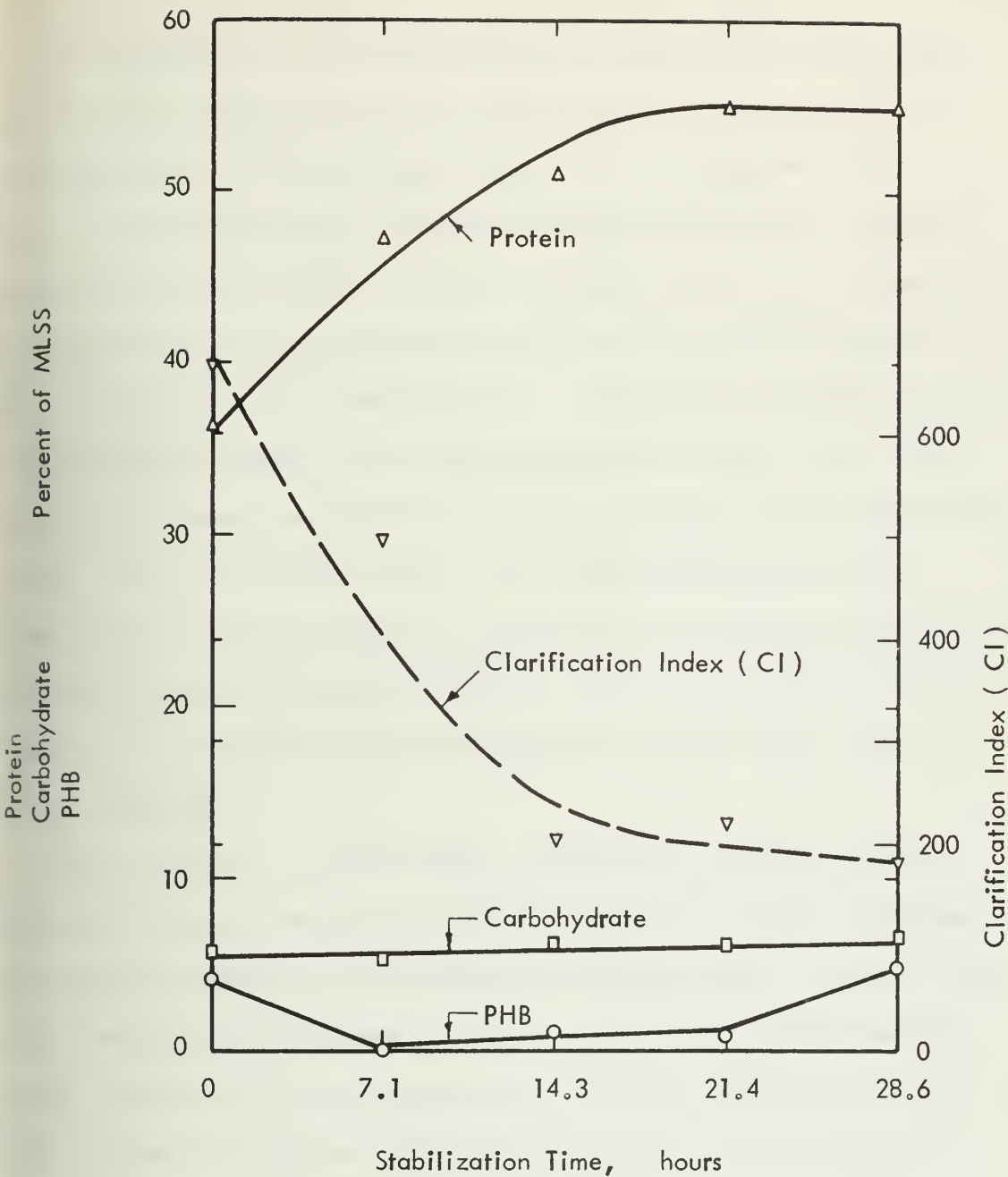
FIGURE 40 VARIATION IN CELLULAR CONSTITUENTS AND CLARIFICATION
INDEX DURING ACTIVATED SLUDGE STABILIZATION IN A
CONTINUOUS FLOW UNIT

An evaluation of the solids-COD/solids ratio of the mixed liquor and the solids-COD/solids ratio of the "other" material provided a good relationship for describing sludge settling characteristics. Figure 41 shows the change in the solids-COD/solids ratio of both the mixed liquor and the "other" material. These data show quite clearly that optimum settling is obtained when the solids-COD/solids ratio of the mixed liquor is at a maximum value. Furthermore, the data indicate that the rate of change in the solids-COD/solids ratio of the "other" material is greater than the over-all rate of change of the solids-COD/ solids ratio of the mixed liquor, thus indicating that the other material is an important factor in determining the settling ability of the sludge. Since the solids-COD/solids ratio of the "other" material changes, it is evident that many compounds are represented by the "other" material.

These findings, in some respect, represent a contradiction of the previous work in which the activity of activated sludge organisms was directly related to the carbohydrate and PHB reserve content of the cell. However, due consideration must be given to the environmental control conditions of cell cultivation in the two systems and their effect on the physiological condition of the cell. It is entirely possible that the environmental conditions imposed in these two experiments caused the apparent differences in the results. In the present study, the cells were grown under conditions similar to those in a chemostat. In fact, the contact aeration tank was a chemostat.

In a chemostat, the cells grow exponentially (98) and they never undergo a period equivalent to the stabilization period that is found in the activated sludge process. The cells used for the fill and draw experiments, on the other hand, were quite different. They were well acclimated to periods of stabilization. The main difference in the two types of cells was in their ability to accumulate carbohydrate and PHB storage products.

Cells acclimated to exponential growth did not accumulate PHB or carbohydrate reserve material whereas cells acclimated to periods of endogenous metabolism (as in the previous batch experiments) did accumulate reserve material. Since the fill and draw method better describes the physiological state of cells in a prototype activated sludge system, it would appear that reserve material is definitely a part of substrate metabolism in the activated sludge process.

Based on the above analysis pertaining to the method of operation for a laboratory activated sludge unit, it is believed that some of the information presented in the present sludge settling study is not relative to the over-all evaluation of storage products in activated sludge systems. However, the data definitely point out the fact that the physical or biochemical properties which influence the settling of activated sludge are not necessarily associated with carbohydrate and lipid storage products. The parameter that correlated the best with settling characteristics was the solids-COD/solids ratio of the MLSS.

VII. SUMMARY DISCUSSION

The existence of poly-beta-hydroxybutyrate (PHB), a unique storage compound, has been demonstrated in laboratory fill and draw activated sludge units. The compound was identified by infrared, carbon-hydrogen-oxygen, melting point and COD analyses. The presence of this material in several activated sludge wastewater treatment plants was indicated through the use of infrared analysis of the solid portion of mixed liquor solids and by the fact that laboratory units, seeded with waste treatment plant mixed liquor, were shown to definitely contain PHB.

A number of different studies of scientific and engineering significance were conducted with activated sludge systems to observe their effect on storage characteristics and composition of the sludge at various stages of growth. The studies were designed to investigate the parameters that influence the quantitative and qualitative nature of storage products in the operation of the process. Loading, variation in nitrogen concentration, and the nature of the feed substrate were considered to be the primary parameters that would quantitatively and qualitatively influence the accumulation of storage material in an activated sludge system. Secondly, the two principal effects of storage compounds on the operation of the activated sludge process, substrate removal and sludge settling, were investigated.

Individual activated sludge units were loaded at rates varying from 0.78 to 7.25 gm COD/(gm MLSS)(day) using a glucose-yeast extract

soluble substrate. It was found that the percentage of substrate converted to carbohydrate and PHB storage products was not dependent on the loading rate up to 4.30 gm COD/(gm MLSS)(day). However, the loading rate did quantitatively affect the per cent by weight of storage products in the cells. As the loading rate increased, the reserve material, both carbohydrate and PHB, represented a greater portion of the solids. What this means is that activated sludge organisms, under the conditions described, will store approximately 60 per cent of the substrate removed, regardless of the loading rate to which they were acclimated, but the resulting weight of storage products constitutes an increasingly larger per cent of the cell weight as the loading is increased up to 4.30 gm COD/(gm MLSS)(day). In all these systems the starting number of organisms, as represented by the weight of protein, was essentially the same (0.3 gm protein/gm MLSS). It was also found that at this loading rate, a minimum amount of oxygen was consumed by the organisms. The combined effect represented an activated sludge system in which a minimum of oxygen was being used and maximum storage was being obtained. Since storage products are degraded more rapidly within the cell than other cellular constituents, the operation at a F/M ratio of 4.30 represents an economically desirable process.

At loadings greater than 4.30 gm COD/(gm MLSS)(day), the carbohydrate storage function began to disappear while protein synthesis, combined with substrate respiration, became the more dominant components of metabolism. Although an increase in substrate

respiration is desirable in some instances of waste treatment, the benefits, in this case, are negated by the fact that protein synthesis and protein degradation both proceed at relatively slow rates. As a result, at F/M ratios higher than 4.30, long periods of aeration would be required thus producing uneconomical operation.

Within the range of COD/N ratios 16.5 to 31.4, the carbohydrate and PHB storage content of the cells increased with increasing COD/N ratio. However, the existence of a maximum COD/N ratio for optimum storage was apparent when a nitrogen deficient unit (COD/N = 56.0) was incapable of storing the lipid polymer. In the same nitrogen deficient unit, there was a 4-hour lag period before carbohydrate storage was observed. Rapid synthesis and rapid degradation of storage materials was exhibited by the cells in the units operated at lower COD/N ratios. It was also found that activated sludge systems operating at a COD/N ratio of 56.0 will satisfactorily remove soluble organic wastes after 25 days of operation. There was a build-up of carbohydrate material during this time but it did not affect the settling characteristics of the sludge.

The composition of the synthetic waste that was fed to different activated sludge units greatly influenced the storage capability of the cell. All laboratory units that did not contain a source of preformed amino acids or peptides did not store PHB. Carbohydrate storage was dependent on the presence of carbohydrate material in the waste feed. Activated sludge systems acclimated to acetic acid as well as glutamic acid did not exhibit any storage

capability whatsoever. With no storage capability it is questionable as to the efficacy of a stabilization or reaeration period in systems that treat wastes of predominantly amino acid or fatty acid nature. Since little or no storage takes place, there would be no reason to reaerate the sludge.

The influence of substrate on the storage ability of cells not only has applicability to waste treatment plants but also to laboratory activated sludge systems. Laboratory units, operated without certain organic nitrogen compounds, could not be expected to retain the original seed organisms that are responsible for PHB synthesis since these organisms have been shown to require preformed organic nitrogen compounds. Therefore, laboratory units which are cultivated on substrates such as acetic acid-ammonium ion complex and glucose-ammonium ion complex would not be expected to undergo the same cellular changes that organisms in an actual treatment plant undergo. Under such circumstances the laboratory units cannot be expected to accurately describe the biology or the biochemistry of the activated sludge process.

From the results of the investigations described above, it appears that substrate storage does not universally occur in activated sludge systems. With a balanced source of organic material, i.e., protein, carbohydrates and lipids, one may expect to find a storage capability in the system. However, as the nature of the substrate becomes more specific, the storage capability becomes qualitatively more specific, and with certain singular substrates, there is no

storage capability at all. When the substrate storage capability is
present in the activated sludge system, its quantitative nature is
dependent primarily on the loading rate and secondarily on the presence
or degree of nitrogen deficiency.

The second phase of investigations was concerned with the
influence of the storage products on the operation of an activated
sludge unit.

The ability of organisms to remove glucose-yeast extract
substrate in a 20-minute contact period was related to the amount of
carbohydrate and PHB reserve material present in cells cultivated in
a batch system. As the carbohydrate and PHB content of the cell
decreased during stabilization, the cells were able to remove greater
quantities of substrate during the 20-minute contact period. When the
carbohydrate and PHB content was reduced to a constant level in the cell,
the amount of substrate removed by the cell was also constant and at a
maximum. The level of storage products in the cell can, therefore,
serve as a measure of the stabilization period required for any activated
sludge system that employs reaeration or stabilization. However,
consideration should also be given to other operational characteristics,
such as sludge settling, in arriving at a minimum stabilization period.
The stored carbohydrate and PHB was not oxidized completely during the
stabilization period. Much of this material was used as substrate for
the synthesis of other cellular compounds. Therefore, in considering
the total loss of substrate through CO_2 evolution, all of the substrate
synthesized into storage material cannot be counted on as being
destroyed in this manner.

In a continuous flow system, with no recirculation of mixed liquor, it was found that no carbohydrates or PHB were stored. The settling characteristics of the activated sludge mixed liquor, after various periods of stabilization, were related more to the oxidation level (solids-COD/solids ratio) of the mixed liquor solids than to the carbohydrate or PHB content of the cell. A solids-COD/solids ratio of 1.30 was required before maximum settling occurred. Below this value the supernatant solids increased as the solids-COD/solids ratio decreased. There existed an obvious discrepancy with relation to the stabilization studies in the fill and draw unit and in the continuous flow unit. In the former system, storage products were degraded and, in the latter system, they were not degraded. However, upon closer examination, this apparent discrepancy may, in fact, be explained on the basis of the physiological condition of the cell and, in turn, on the cultivation technique that was used. In the fill and draw unit there was always a certain percentage of old cells that had been recirculated from a starvation environment before being subjected to a new food source. On the other hand, the continuous flow system (chemostat) produced cells that had never undergone starvation, and thus would not be equipped with the same enzyme complement as the cells obtained from the fill and draw unit. Thus it appears that the storage capability in an activated sludge system, since it was found only in the fill and draw unit, must be intimately associated with metabolic activities related to a state of endogenous respiration. Had the cells in the continuous flow unit been subjected to a period

of starvation and then recycled into an environment containing an excess of food, it is believed that a storage function would have been demonstrated. Since the fill and draw unit closely approximates conditions that would be found in an activated sludge wastewater treatment plant, it is believed the data relating the storage capability to stabilization period are more reliable from that unit than from the flow through unit.

It should also be pointed out that caution must be exercised during the development of laboratory models for use in activated sludge research. If at all possible, the activated sludge physical model should be designed such that the bacterial model of the waste treatment plant is duplicated in the laboratory.

VIII.   CONCLUSIONS

1. Poly-beta-hydroxybutyrate (PHB), a unique storage compound, can
    be isolated from a laboratory activated sludge unit that has been
    seeded with municipal wastewater treatment plant activated sludge.

2. The presence of poly-beta-hydroxybutyrate in activated sludge from
    municipal wastewater treatment plants may be assumed to be present
    through the use of infrared analysis of the activated sludge
    solids.

3. The loading rate (F/M ratio) of an activated sludge unit <u>does</u> <u>not</u>
    influence the amount of substrate that is converted to storage
    products up to an F/M ratio of 4.30 gm COD/(gm MLSS)(day).
    Approximately 60 per cent of a soluble substrate is converted to
    storage products within the loading range of 0.78 to 4.30
    gm COD/(gm MLSS)(day).

4. The loading rate (F/M ratio) to which an activated sludge unit is
    acclimated, <u>does</u> influence the per cent of storage products in
    the mixed liquor solids. As the loading rate increases up to
    F/M 4.30, the newly synthesized storage products constitute an
    increasingly larger per cent of the cell weight. Beyond F/M 4.30
    a greater percentage of the substrate is used for protein
    synthesis.

5. The COD/N ratio to which an activated sludge unit is acclimated,
    will only slightly influence the amount of storage products that
    can be accumulated by the mixed liquor solids. Up to a COD/N

ratio of 31.4, the storage products increase with increasing COD/N ratio. In a nitrogen deficient unit, COD/N ratio of 56.0, the organisms do not accumulate PHB while carbohydrate accumulation is maintained at the same level as for a unit acclimated at a COD/N ratio of 31.4.

6. The nature of the storage compound, carbohydrate or PHB, is significantly influenced by the nature of the waste. An activated sludge unit, acclimated to a carbohydrate-ammonium ion substrate will accumulate only carbohydrate storage material while a unit acclimated to a fatty acid-ammonium ion substrate or an amino acid substrate, will not accumulate any storage material. The accumulation of PHB appears to be dependent on the presence of preformed amino acids in addition to a carbohydrate food source.

7. In a fill and draw operated activated sludge system undergoing stabilization, the ability of the stabilized sludge to remove substrate is related to the amount of storage material within the cell. As the storage material decreases, the cells have a greater ability to remove substrate.

8. The use of a continuous flow laboratory activated sludge unit (chemostat) does not appear to be an appropriate model to use for investigating the parameters that influence the activated sludge process. This is particularly true in studies related to the cellular composition of the mixed liquor because the organisms appear to be physiologically different than those

organisms which are periodically exposed to the conditions of endogenous respiration as might be found in the stabilization period of the activated sludge process.

IX. SUGGESTIONS FOR FUTURE WORK

In view of the results obtained from this study, it is
suggested that certain portions of this investigation be extended to
include the following:

1. A study to determine if a relationship exists between the infrared
   absorption spectrum of activated sludge from a municipal wastewater
   treatment plant and the settling characteristics of the sludge. In
   the present study, the activated sludge obtained from the municipal
   treatment plant at Mattoon exhibited an exceptionally strong
   absorption peak at 5.8 microns. This sludge also had very poor
   settling characteristics.

2. Re-evaluate the influence of storage products, such as PHB, on the
   settling characteristics of activated sludge using a laboratory
   model that closely describes the metabolic activities of the full
   size municipal treatment plant. Such a model should be continuous
   flow and include recirculation of the sludge.

3. Thoroughly investigate the biochemical changes in the composition
   of activated sludges acclimated to amino acids. Little or no change
   in composition, such as found in the present studies, may provide
   the basis for further determining if a stabilization period is
   needed for activated sludges which treat such wastes.

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<u>Education</u>:

February 1948 - Graduated from High School

June 1954 - B.C.E.,., University of Dayton

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<u>Brief Chronology of Employment</u>:

1953 - 1955 U. S. Army, Corps of Engineers

1955 - 1957 Structural Engineer, Milwaukee, Wisconsin

1957 - 1959 Graduate Research Assistant, Sanitary Engineering

Department, Purdue University

1959 - Present U. S. Public Health Service

A) Arctic Health Research Center, Project Engineer
(1959-1962)

B) Outside-The-Service Training (1962-1965)

C) Basic and Applied Sciences Branch, DWSPC,
Technical Assistant to the Chief (1965-Present)

<u>Societies</u>:

American Water Works Association

American Society for Microbiology

American Institute of Biological Sciences

Societies (continued)

American Society of Civil Engineers

Water Pollution Control Federation

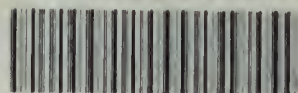Honors & Other Special Scientific Recognition:

Tau Beta Pi

Sigma Xi

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Publications:

1. "Sewage Lagoons in Alaska," Proc., 10th Alaskan Science
   Conference, Alaska Division, AAS, 1959.

2. "Eskimo Algae Make Lagoons Work at the Arctic Circle,"
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